RNeasy® 96 Handbook

For RNA isolation from animal and human cells

and for RNA cleanup

January 2002

QIAGEN Worldwide

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Contents

Kit Contents

Kit	RNeasy® 96 Kit (4)	RNeasy 96 Kit (12)
Catalog No.	**74181**	**74182**
Preparations per kit	4 x 96	12 x 96
RNeasy 96 Plates	4	12
Register Cards (96-well)	4	12
Square-Well Blocks (2.2 ml)*	2	2
Elution Microtubes (1.2 ml), racked	4 x 96	12 x 96
Caps for Elution Microtubes, in strips	55 x 8	165 x 8
AirPore™ Tape Sheets	3 x 5 sheets	2 x 25 sheets
Buffer RLT†	220 ml	2 x 220 ml
Buffer RLN	45 ml	3 x 45 ml
Buffer RW1†	2 x 220 ml	4 x 400 ml
Buffer RPE‡	4 x 55 ml	8 x 65 ml
RNase-free water	2 x 50 ml	12 x 30 ml
Handbook	1	1

* *Reusable; see page 17 for cleaning instructions.*

† *Not compatible with disinfecting containing bleach. Contains guanidine isothiocyanate, which is an irritant. Take appropriate safety measures, and wear gloves when handling.*

‡ *Buffer RPE is supplied as a concentrate. Add 4 volumes of ethanol (96–100%) before use to obtain a working solution of Buffer RPE.*

Additional Buffer RLT, Buffer RLN, Square-Well Blocks, Elution Microtubes (1.2 ml), and AirPore Tape Sheets are available separately. See ordering information (page 57).

Storage Conditions

RNeasy 96 Kits should be stored dry, at room temperature, and are stable for at least 9 months under these conditions. Buffer RLN should be precooled for use in the cytoplasmic protocol and can be stored at 2–8°C if desired.

Product Use Limitations

Some QIAGEN® products may be used in clinical diagnostic laboratory systems after the laboratory has validated their complete systems as required by CLIA '88 regulations in the U.S. or equivalents in other countries. All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see inside front cover).

Technical Assistance

At QIAGEN we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in molecular biology and the use of QIAGEN products. If you have any questions or experience any difficulties regarding the RNeasy 96 Kit or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information please call one of the QIAGEN Technical Service Departments or local distributors (see inside front cover).

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/ts/msds.asp** where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

CAUTION: DO NOT add bleach or acidic solutions directly to the sample-preparation waste.

Buffers RLT and RW1 contain guanidine thiocyanate, which can form highly reactive compounds when combined with bleach.

If liquid containing these buffers is spilt, clean with suitable laboratory detergent and water. If the spilt liquid contains potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 1% (v/v) sodium hypochlorite.

The following risk and safety phrases apply to the components of the RNeasy 96 Kit:

Buffer RLT

Contains guanidine thiocyanate: harmful. Risk and safety phrases:* R20/21/22-32 S13-26-36-46

Buffer RW1

Contains ethanol: flammable. Risk and safety phrases:* R10

24-hour emergency information

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

** R10: flammable; R20/21/22: Harmful by inhalation, in contact with skin and if swallowed.R32: Contact with acids liberates very toxic gas; S13: Keep away from food, drink and animal feedingstuffs; S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice;S36: Wear suitable protective clothing; S46: If swallowed, seek medical advice immediately and show this container or label.*

Introduction

The RNeasy 96 Kit is ideal for simultaneous isolation of 96 or 192 RNA samples from up to 5 x 10^5 animal or human cells per sample. The RNeasy 96 Kit facilitates efficient, high-throughput RNA sample preparation for research use (see Product Use Limitations, page 5) in fields such as:

- Drug screening
- Molecular diagnostics
- Therapy monitoring
- Basic research

In less than 1 hour, 192 high-purity RNA samples can be obtained when processing 2 RNeasy 96 plates in parallel (15–20 s per RNA sample). The RNeasy 96 procedure replaces current time-consuming and tedious methods involving alcohol-precipitation steps, large numbers of washing steps, or the use of toxic substances such as phenol and/or chloroform. The purified RNA is ready to use in any downstream application including:

- RT-PCR
- Quantitative RT-PCR, including TaqMan® and LightCycler® technology
- Differential display
- cDNA synthesis
- Northern, dot, and slot blot analysis
- Primer extension
- Poly A^+ RNA selection
- RNase/S1 nuclease protection
- Microarrays

In addition, the RNeasy 96 Kit can be used to desalt or to purify RNA from enzymatic reactions such as DNase digestions, proteinase digestions, RNA ligation, or labeling reactions.

The RNeasy 96 principle and procedure

The RNeasy 96 Kit represents a new technology for high-throughput RNA preparation. This technology combines the selective binding properties of a silica-gel–based membrane with the speed of vacuum and/or spin technology. Cells are first lysed under highly denaturing conditions with guanidine isothiocyanate (GITC) to immediately inactivate RNases and ensure isolation of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to the wells of the RNeasy 96 plate. Total RNA binds and contaminants are efficiently washed away. High-quality RNA is then eluted in a small volume of water, ready for use in any downstream application.

With the RNeasy 96 procedure (Figure 1), all RNA molecules longer than 200 nucleotides are isolated. The procedure provides an enrichment for mRNA since most RNAs <200 nucleotides (such as 5.8S rRNA, 5S rRNA, and tRNAs, which together comprise 15–20% of total RNA) are selectively excluded. The size distribution of purified RNA is comparable to that obtained by centrifugation through a CsCl cushion, where small RNAs do not sediment efficiently.

Different protocols are provided with different lysis steps and different handling options to pass solutions through the membrane using vacuum and/or spin technology (see page 11 for a detailed description).

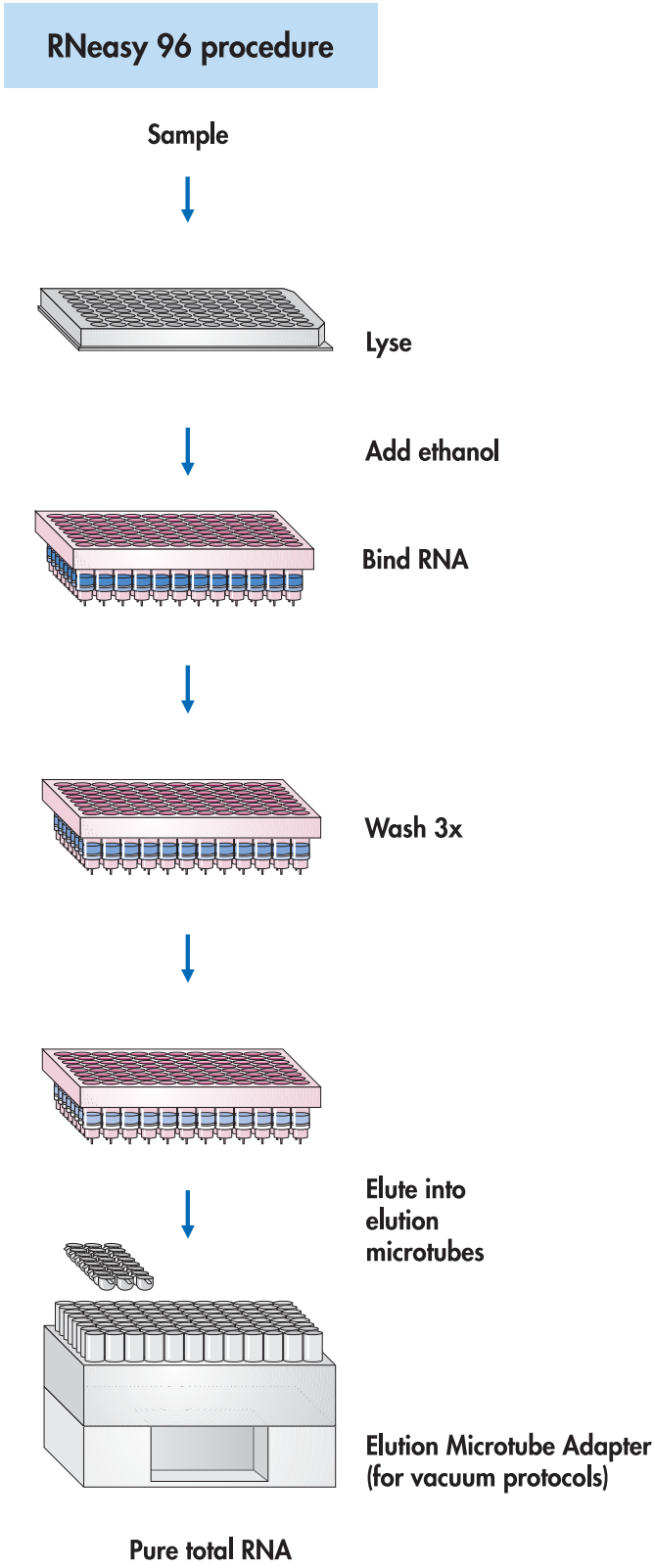

Figure 1. *RNA isolation with the RNeasy 96 Kit. Protocol steps can be performed on a QIAvac 96 vacuum manifold (with the Elution Microtube Adapter shown) or in a specially designed centrifuge system (see page 11).*

Description of protocols

Isolation of Total RNA from Animal Cells

The RNeasy 96 procedure is optimized for processing up to 5 x 10^5 animal cells per sample (see "Sample size", page 12 for details). In the **RNeasy 96 Protocol for Isolation of Total RNA from Animal Cells** (page 19), cells are lysed in a buffer containing guanidine isothiocyanate (GITC). Ethanol is then added to the lysates, creating conditions that promote selective binding of RNA to the RNeasy membrane. The samples are then applied to the wells of the RNeasy 96 plate. Total RNA binds to the membrane at the bottom of each well. Contaminants are efficiently washed away by wash buffers, and the RNeasy membrane is dried. High-quality RNA is then eluted in a small volume of water.

Isolation of Cytoplasmic RNA from Animal Cells

The **RNeasy 96 Protocol for Isolation of Cytoplasmic RNA from Animal Cells** (page 26) is optimal for applications where unspliced or partially spliced RNA is not desirable, since the cytoplasm contains RNA in its mature form. The protocol is also advantageous in applications where the absence of DNA contamination is critical, since the intact nuclei are removed (see also "DNA contamination", page 48). Using the cytoplasmic protocol, DNase digestion is generally not required: most of the DNA is removed with the nuclei during the procedure, and the RNeasy 96 silica-membrane technology efficiently removes nearly all of the remaining small amounts of DNA without DNase treatment.

Cultured cells are lysed in a buffer containing the non-ionic detergent Nonidet® P-40, which lyses the cell plasma membrane. Nuclei remain intact during the lysis procedure and are removed by centrifugation. GITC-containing lysis buffer and ethanol are added to the supernatant to provide optimal conditions for selectively binding RNA to the RNeasy membrane. Cytoplasmic RNA binds to the membrane at the bottom of each well. The optional on-column DNase step allows digestion of remaining DNA. DNase and contaminants are efficiently washed away by wash buffers, and the RNeasy membrane is dried. High-quality RNA is then eluted in a small volume of water.

RNA Cleanup

The **RNeasy 96 Protocol for RNA Cleanup** (page 35) is used to purify RNA from enzymatic reactions (e.g., RNA labeling, DNase digestion) or for desalting RNA samples (maximum 100 µg of RNA per well). GITC-containing lysis buffer and ethanol are added to the sample to create conditions that promote selective binding of RNA to the RNeasy membrane. The samples are then applied to the wells of the RNeasy 96 plate. RNA binds to the membrane at the bottom of each well. Contaminants are efficiently washed away by wash buffers, and the RNeasy membrane is dried. High-quality RNA is then eluted in a small volume of water.

Handling options

Each of the protocols in this handbook is provided with three different handling options, using vacuum technology, a combination of vacuum and spin technology, or spin technology alone. Each handling option provides high yields of high-quality RNA. The requirements of the downstream application determine which option should be used.

I. Vacuum technology

Using the QIAvac 96 vacuum manifold (see pages 14–15) is the quickest way to carry out the RNeasy 96 RNA isolation. Up to 96 RNA samples can be processed in 30–40 minutes. Use of two vacuum manifolds to process two RNeasy 96 plates in parallel allows processing of up to 192 RNA samples in 35–45 minutes. RNA isolated using vacuum technology can be used in any non-enzymatic application (e.g., northern, dot, and slot blot analysis). The RNA may also be used in enzymatic applications. However, because RNA samples prepared using vacuum technology may still contain trace amounts of salt, we recommend preliminary experiments with the application required. If RNA performance is unsatisfactory, the RNeasy 96 vacuum/spin or spin options should be used.

II. Vacuum/spin technology

Using vacuum/spin technology, all protocol steps up to the final wash step are performed on the QIAvac 96 vacuum manifold (see pages 14–15). The final wash step, including membrane drying, and all the elution steps are performed in the Centrifuge 4-15C or Centrifuge 4K15C (see page 16). The Plate Rotor 2 x 96 holds two RNeasy 96 plates, allowing up to 192 RNA samples to be prepared in only 1 hour. Residual traces of salt are removed by centrifugation in the final wash step. RNA isolated using vacuum/spin technology can be used for any non-enzymatic or enzymatic downstream application including quantitative RT-PCR analysis by TaqMan technology.

III. Spin technology

Using spin technology, all protocol steps are performed in the Centrifuge 4-15C or Centrifuge 4K15C (see page 16). The Plate Rotor 2 x 96 holds two RNeasy 96 plates, allowing up to 192 RNA samples to be prepared in only 1.5 hours. RNA isolated using spin technology can be used for any non-enzymatic or enzymatic downstream application including quantitative RT-PCR analysis by TaqMan technology.

Important Notes before Using the RNeasy 96 Kit

Sample size

The RNeasy 96 procedure is optimized for use with 10* to 5 x 10^5 animal cells. Direct counting is the most accurate way to quantify the number of cells. However Table 1 may be used as a guide.

Table 2 gives specifications for the RNeasy 96 plate. Each well of the RNeasy 96 plate has a maximum binding capacity of 100 µg of RNA, but actual yields depend on the sample. The RNA amounts for the recommended numbers of cells are significantly less than the binding capacity of the RNeasy membrane and expected yields are therefore less than the RNeasy 96 binding capacity. Table 3 gives examples of expected RNA yields from various cultured animal cells.

If more than 5 x 10^5 cells are to be processed, the volume of lysis buffer and other reagents added to the sample before loading must be doubled. The volumes of the wash and elution buffers need not be increased. Depending on the cell line, the lysates may become viscous when starting with more than 5 x 10^5 cells. This may lead to significantly lower yield and purity and may cause the RNeasy 96 plate wells to clog. If using more than 5 x 10^5 cells, we recommend performing preliminary experiments, increasing the number of cells step-by-step (e.g., 5 x 10^5, 7.5 x 10^5, 1 x 10^6 cells) and analyzing RNA yield and purity for each cell number. More than 1 x 10^6 cells should not be processed as lysates become too viscous.

Note: Additional Buffer RLT can be purchased separately (see ordering information on page 57).

Table 1. Growth area and number of HeLa cells in various multiwell cell-culture plates

Cell culture vessel	Growth area (cm^2)†	Number of cells‡
Multiwell plates		
96-well	0.32–0.60	4–5 x 10^4
48-well	1.0	1.3 x 10^5
24-well	2.0	2.5 x 10^5
12-well	4.0	5.0 x 10^5
6-well	9.5	1.2 x 10^6

† *Growth area varies slightly depending on the supplier. Values are reported per well.*

‡ *Confluent growth is assumed. Values are reported per well.*

* *Please call QIAGEN Technical Services for guidelines to purify RNA from 10–100 cells.*

Table 2. RNeasy 96 plate specifications

Preps per plate	96
Amount of starting material	10 to 5 x 10^5 cells*
Binding capacity per well	100 µg RNA†
Maximum loading volume per well	1 ml
RNA size distribution	All RNA >200 nucleotides

* *The RNeasy 96 procedure is optimized for processing up to 5 x 10^5 animal cells. Depending on the cells used it may be possible to increase the maximum amount of starting cells up to 1 x 10^6 cells (see text). Please call QIAGEN Technical Services for guidelines to purify RNA from 10–100 cells.*

† *Yields are limited by cell type and number. The maximum binding capacity of 100 µg RNA is usually not reached (see text).*

Table 3. Average total RNA yields obtained from a variety of cell lines using the RNeasy 96 Kit

Animal cell line	Source	Yield‡ (µg) for 1 x 10^5 cells
HeLa	Human cervical carcinoma	1.6
LMH	Chicken hepatoma	1.3
COS-7	Monkey kidney, SV-40 transformed	3.1
Huh7	Human hepatoma	2.0
Jurkat	Human T-cell leukemia	1.4
K-562	Human chronic myelogenous leukemia in blast crisis	1.9

‡ *Amounts can vary due to development stage, growth conditions used, etc. Since the RNeasy procedure enriches for RNA >200 bases long, the total RNA yield does not include 5.8S rRNA, tRNA, and other low-molecular weight RNAs, which make up 15–20% of total cellular RNA.*

Handling and storage of starting material

RNA is not protected until the sample material is flash frozen or disrupted in the presence of RNase-inhibiting or denaturing agents. It is therefore important that cell samples are immediately frozen and stored at –70°C or processed as soon as harvested. The relevant procedures should be carried out as quickly as possible. Samples can also be stored at –70°C in lysis buffer (RLT) after disruption. Frozen samples are stable for months.

Note: For isolation of cytoplasmic RNA from animal cells, only freshly harvested cells can be used.

QIAvac 96 Vacuum Manifold

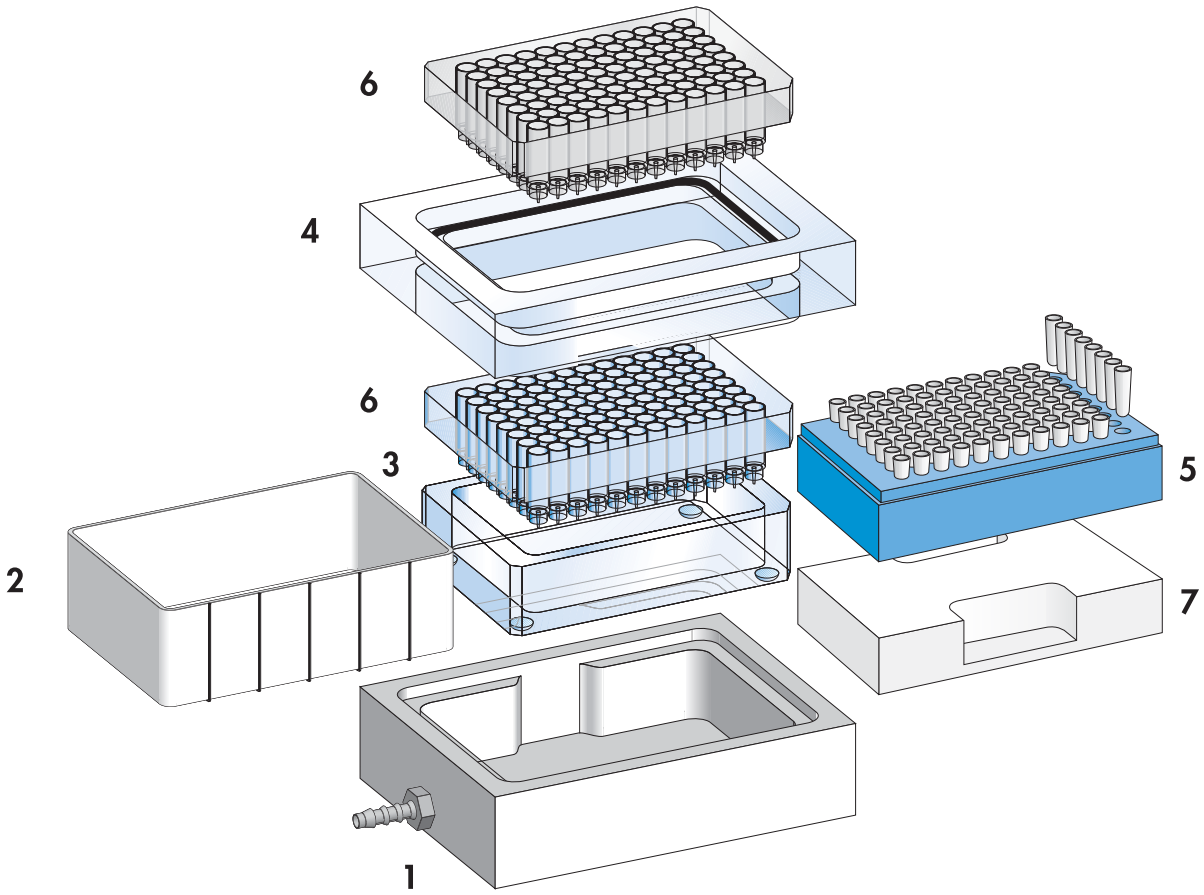

Figure 2. *Components of the QIAvac 96 vacuum manifold*

1. *QIAvac base, which holds a waste tray, a plate holder, or a microtube rack*
2. *Waste tray*
3. *Plate holder (shown with 96-well plate) — not used in RNeasy 96 Protocol*
4. *QIAvac 96 top plate with aperture for 96-well plate*
5. *Disposable microtube rack**
6. *96-well plate**
7. *Elution Microtube Adapter**

** Not included with QIAvac 96.*

QIAvac 96 handling guidelines

QIAvac 96 facilitates RNeasy minipreparation by providing a convenient, modular vacuum manifold (Figure 2) for use with the RNeasy 96 Kit. The following recommendations should be followed when handling the QIAvac 96 vacuum manifold.

- QIAvac 96 operates with house vacuum or a vacuum pump. If the house vacuum is weak or inconsistent, we recommend using a vacuum pump with a capacity of 18 liter/min. Use of insufficient vacuum pressure may reduce RNA yield and purity.
- A vacuum pressure of –800 to –900 mbar should develop when an RNeasy 96 plate sealed with tape is used on the QIAvac 96. Vacuum pressures exceeding –900 mbar should be avoided. The vacuum pressure is the pressure differential between the inside of the manifold and the atmosphere (standard atmospheric pressure: 1013 millibar or 760 mm Hg) and can be regulated and measured using a pressure gauge or vacuum regulator (see ordering information, page 57). Vacuum recommendations are given in negative units to indicate the required reduction in pressure with respect to the atmosphere. Table 4 provides pressure conversions to other units.
- Between loading steps, the vacuum must be switched off and the manifold ventilated to maintain uniform conditions for each sample. This can be done with a vacuum regulator (see ordering information, page 57) inserted between the vacuum source and the QIAvac 96 vacuum manifold.
- Wear safety glasses when working near a manifold under pressure.
- Always place the RNeasy 96 plate into the vacuum manifold with the beveled edges pointing to the right-hand side. For safety reasons, do not use plates that have been damaged in any way.
- Always place the QIAvac 96 vacuum manifold on a secure bench top or work area. If dropped, the manifold may crack.
- Always store the QIAvac 96 vacuum manifold clean and dry. To clean, simply rinse all components with water, and dry with paper towels. Do not air dry, as the screws may rust and need to be replaced. Do not use abrasives. Finally, wipe manifold components with paper towels wetted with 70% ethanol, and dry with fresh paper towels.
- The QIAvac 96 vacuum manifold and components are not resistant to ethanol, methanol, or other organic solvents when exposed for long periods. If solvents are spilled on the unit, rinse thoroughly with distilled water after the RNeasy preparation. Ensure that no residual buffers remain in the vacuum manifold.
- To ensure consistent performance, do not apply silicone or vacuum grease to any part of the QIAvac 96 vacuum manifold. The spring lock on the top plate and the self-sealing gasket provide an airtight seal when the vacuum is applied to the assembled unit. To maximize gasket life, rinse the gasket free of salts and buffers after each use, and dry with paper towels before storage.

Table 4. Pressure conversions

To convert from millibars (mbar) to:	Multiply by:
Millimeters of mercury (mm Hg)	0.75
Kilopascals (kPa)	0.1
Inches of mercury (inch Hg)	0.0394
Torrs (Torr)	0.75
Atmospheres (atmos)	0.000987
Pounds per square inch (psi)	0.0145

Centrifuge 4-15C and Centrifuge 4K15C

RNeasy 96 protocols using vacuum/spin and spin technology utilize a streamlined centrifugation procedure that allows preparation of RNA from up to 2 x 96 samples in parallel for direct use in any downstream application. For optimal handling, QIAGEN, in cooperation with the centrifuge manufacturer Sigma Laborzentrifugen GmbH, has developed a centrifugation system consisting of the Plate Rotor 2 x 96, and the table-top Centrifuge 4-15C or Centrifuge 4K15C (see ordering information, page 57). A wide range of other rotors can be used with Centrifuge 4-15C or Centrifuge 4K15C in addition to the Plate Rotor 2 x 96.

Standard table-top centrifuges and 96-well–microplate rotors are not suitable in the RNeasy 96 procedure. Usually 96-well–microplate buckets are not deep enough to carry the complete RNeasy assembly without interfering with how the buckets swing out. Furthermore, high *g*-forces (>5500 x *g*) are required for optimal performance of RNeasy 96.

Centrifuge 4-15C and Centrifuge 4K15C are suitable for isolating total RNA and, in most cases, cytoplasmic RNA. However, when isolating cytoplasmic RNA from RNase-rich cells, it may be necessary to pellet the nuclei at 4°C to avoid RNA degradation. This step can be performed in either a standard refrigerated 96-well–microplate centrifuge or Centrifuge 4K15C, which can also be used for all RNeasy 96 spin steps (see ordering information, page 57).

Note: If Centrifuge 4K15C is used, set the temperature at room temperature (20 to 30°C) for all remaining centrifugation steps.

For further information about the centrifuges and rotor please contact QIAGEN or your local distributor.

Abbreviated instructions for using the Centrifuge 4-15C

1. Switch on the centrifuge by pressing the main switch on the back.
2. Select the rotor selection list in the display field by turning the knob. After pressing the knob, turn the knob again to select the rotor/bucket combination "09100/09158" for the Plate Rotor 2 x 96. Confirm entry by pressing the knob. Entering the rotor number automatically sets the time and speed limits for centrifugation for that particular rotor, eliminating the danger of the centrifuge overspeeding.
3. Select "Speed" by turning the knob. Press the knob, and set the speed to "6000" by turning the knob again. Confirm entry by pressing the knob. The corresponding relative centrifugal force (RCF) is calculated from the rotor number and speed and appears automatically in the RCF field. It is also possible to enter the RCF value "5788 x *g*" manually in the RCF field after selecting "RCF" in the same way.
4. Select "Time" by turning the knob. Press once, and by turning the knob again, set the time as recommended in the particular protocol step. Confirm entry by pressing the knob.
5. Open the lid, place the 96-well plates with the metal carriers in the buckets, and close the lid. The start and lid keys light up.
6. Push the start key to start the centrifuge. When the centrifuge is running the lid key will not be lit. Each run can be interrupted by pushing Stop.
7. At the end of the run, the lid key will light up. Open the centrifuge lid by pressing the lid key. Remove the plates. All preset parameters remain after a run has finished.

Warning: Do not centrifuge the Plate Rotor 2 x 96 metal holders without the RNeasy 96 plates and Square-Well Blocks or elution microtubes. If unsupported, the holders will collapse under high *g*-force. Therefore, remove the holders during test runs. Standard 96-well microplates may be centrifuged in the holders if a *g*-force of 500 x *g* is not exceeded.

Square-Well Blocks

Four Square-Well Blocks are supplied per kit. If several RNeasy 96 plates are processed per day, it may be convenient to keep extra Square-Well Blocks on hand. See ordering information on page 57.

To reuse the Square-Well Blocks, rinse them thoroughly with tap water, incubate for 2 h or overnight in 0.1 N NaOH/1 mM EDTA, rinse in distilled water, and dry at 50°C.

Note: Do not use bleach. Bleach may react with residual amounts of Buffers RLT and RW1 on the Square-Well Blocks.

Reagents and equipment to be supplied by user

For all protocols

- Multichannel pipet with tips
  For the most efficient sample processing in the RNeasy 96 protocol, we recommend the use of an electric multichannel pipet with a minimum capacity of 650 µl per pipet tip. Good options are the Matrix Impact® cordless electronic multichannel pipet or the Matrix Multi-8 Electrapette®, both of which have a unique expandable tip-spacing system allowing the user to transfer liquid directly from racks of tubes to 96-well microplates. See Appendix F (page 56) for ordering information.
- Reagent reservoirs for multichannel pipets
- Disposable gloves
- Square-Well Blocks (Cat. No. 19573, optional)*
- 96–100% ethanol
- 14.5 M β-mercaptoethanol (β-ME, optional)†
- **Using vacuum technology**
  - QIAvac 96 vacuum manifold (see pages 14–15)
  - Vacuum source capable of generating a vacuum pressure of –800 to –900 mbar (see page 15)
  - Elution Microtube Adapter (available from QIAGEN Technical Services)
- **Using vacuum/spin technology**
  - Centrifuge 4-15C or Centrifuge 4K15C (see page 16)
  - Plate Rotor 2 x 96 (see page 16)
  - QIAvac 96 vacuum manifold (see pages 14–15)
  - Vacuum source capable of generating a vacuum pressure of –800 to –900 mbar (see page 15)
- **Using spin technology**
  - Centrifuge 4-15C or Centrifuge 4K15C (see page 16).
  - Plate Rotor 2 x 96 (see page 16)

** Four Square-Well Blocks are supplied with the kit. They can be reused (see page 17). If several plates are processed per day it may be convenient to have extra Square-Well Blocks available.*

† Addition of β-ME to Buffer RLT is optional for the RNeasy 96 protocols (see protocols for detailed information).

For protocol to isolate total RNA

- 70% ethanol in water

For protocol to isolate cytoplasmic RNA

- Centrifuge and rotor for 96-well microplates (see page 16)
- RNase inhibitor (optional)*
- DTT (optional)*

For optional DNase treatment on RNeasy 96 plates

Generally, DNase digestion is not required since the RNeasy 96 silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundant target). In these cases, an optional on-column DNase step allows digestion of the small residual amounts of DNA remaining. The DNase is efficiently removed in the following wash step of the protocol.

- DNase I, RNase-free, ≥1.8 Kunitz units/µl

  The amount of DNase I used in the protocols is given in Kunitz units. A Kunitz unit is a commonly used unit for measuring DNase I, defined as the amount of DNase I that causes an increase in A_{260} of 0.001 per minute per milliliter at 25°C, pH 5.0, with highly polymerized DNA as the substrate (Kunitz, M. (1950) J. Gen. Physiol. **33**, 349 and 363).

  Note: Some suppliers have different unit definitions for DNase I. For example, 1 unit of DNase I from Pharmacia will degrade 1 µg of pBR322 in 10 minutes at 37°C at pH 7.5. One Pharmacia unit is equal to approximately 0.3 Kunitz units. **Make sure to use the appropriate conversion factor for DNase I preparations that are not quantified in Kunitz units.**

- Buffer RDD from QIAGEN, optimized for DNase I digestion on the RNeasy membrane (available from QIAGEN upon request)

  Note: Standard DNase buffers are not compatible with on-membrane DNase digestion. Use of other buffers may affect the binding of the RNA to the RNeasy membrane, reducing the yield and integrity of the RNA.

- Prepare DNase I incubation mix immediately before starting the RNeasy 96 protocol. Prepare the mix according to the table below. Vortex briefly and keep on ice until use.

Component	Amount/96-well plate
DNase I, RNase-free	1800 Kunitz units (maximum 1.0 ml)†
Buffer RDD‡	7.0 ml
RNase-free water, supplied in the RNeasy 96 Kit	Add RNase-free water to 8.0 ml if necessary

† *Make sure to use a DNase I solution with ≥1.8 Kunitz units/µl. Use of more than 1.0 ml will not provide optimal reaction conditions due to high concentrations of glycerol, which is commonly used in DNase I storage buffers. Use the appropriate conversion factor for DNase I preparations that are not quantified in Kunitz units (see above).*

‡ *Available from QIAGEN upon request. Standard DNase buffers are not compatible with on-membrane DNase digestion. Use of other buffers may affect the binding of the RNA to the RNeasy membrane, reducing the yield and integrity of the RNA.*

* *Addition of RNase inhibitor and DTT to Buffer RLN is optional (see RNeasy 96 Protocol for Isolation of Cytoplasmic RNA from Animal Cells, page 26).*

RNeasy 96 Protocol for Isolation of Total RNA from Animal Cells

Important notes before starting

- If preparing RNA for the first time, please read Appendix A (page 45). If using the RNeasy 96 Kit for the first time, please read ''Important Notes before Using the RNeasy 96 Kit'' (page 12).
- All centrifugation steps in the vacuum/spin protocol and in the spin protocol are performed in a Centrifuge 4-15C or Centrifuge 4K15C (see page 16).
- Use of a multichannel pipet is recommended (see page 17). Pour buffers and RNase-free water into reagent reservoirs for multichannel pipets. Use reservoirs from a freshly opened package or clean them as described for Square-Well Blocks (see page 17).
- A vacuum source capable of generating a vacuum pressure of –800 to –900 mbar is necessary for the vacuum and vacuum/spin protocol (see page 15). The vacuum must be switched off and the manifold ventilated between pipetting steps to maintain uniform conditions for each sample.
- Cell pellets can be stored at –70°C for later use or used directly in the procedure. Frozen cell pellets should be thawed slightly. Continue with steps 1 and 3 (using vacuum or vacuum/spin technology) or with step 2 (using spin technology).
- Cell lysates in Buffer RLT can be stored at –70°C for several months. To process frozen lysates, thaw and incubate at 37°C for 10 min to ensure that the chaotropic salt has dissolved. Mix by pipetting up and down 3 times. Continue with steps 1 and 4 (using vacuum or vacuum/spin technology) or with step 3 (using spin technology).
- Buffer RLT may form a precipitate upon storage. If necessary, warm to 37°C to redissolve.
- When isolating RNA from cells containing high amounts of RNases, it may be necessary to add β-mercaptoethanol (β-ME) to Buffer RLT to avoid degradation of RNA. β-ME supports the inactivation of RNases by GITC. Add 10 µl of 14.5 M β-ME per 1 ml of Buffer RLT. Buffer RLT is stable for 1 month after addition of β-ME. In most cases it will not be necessary to add β-ME to Buffer RLT.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) to obtain a working solution.
- All steps of the RNeasy 96 protocol for isolation of total RNA should be performed at room temperature (20 to 30°C). Avoid interruptions during the procedure.
- Generally, DNase digestion is not required since the RNeasy 96 silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA. See page 48 for more information and page 18 for details to prepare the DNase I incubation mix.

RNeasy 96 Protocol for Isolation of Total RNA from Animal Cells

I. Using vacuum technology

1. Preparation of QIAvac 96 vacuum manifold

Place the waste tray inside the QIAvac base. Place the top plate squarely over the base. Place the RNeasy 96 plate in the QIAvac top plate, making sure that the plate is seated tightly. Attach QIAvac 96 manifold to the vacuum source. Keep vacuum switched off.

Note: Always place the RNeasy 96 plate into the vacuum manifold with the beveled edges pointing to the right-hand side.

2. Harvesting cells

a) Cells grown in a monolayer
Cells grown in a monolayer in multiwell cell-culture plates can be lysed directly in the wells. Completely remove medium by pipetting, and continue with step 3.

Note: Incomplete removal of the supernatant will dilute Buffer RLT in subsequent steps, inhibiting lysis and the binding of RNA to the RNeasy membrane. This will lead to reduced yield.

b) Cells grown in suspension
Transfer aliquots of up to 5 x 10^5 cells into the wells of a 96-well microplate. Spin cells for 5 min at 300 x *g*, and completely remove supernatant by pipetting. Proceed with step 3.

Note: Incomplete removal of the supernatant will dilute Buffer RLT in subsequent steps, inhibiting lysis and the binding of RNA to the RNeasy membrane. This will lead to reduced yield.

3. Add 150 µl of Buffer RLT to each microplate well. Keeping the microplate flat on the bench, shake it vigorously back and forth for 10 s. While continuing to keep the plate flat on the bench, rotate the plate by 90° and shake it for an additional 10 s.

Note: After the addition of ethanol in step 4, total volume will be 300 µl. If the surface of the liquid is too high for convenient use in the 96-well microplate used, the volume of Buffer RLT may be reduced to 100 µl.

4. Add 1 volume (150 µl) of 70% ethanol. Mix by pipetting up and down 3 times.

Note: Add 100 µl of 70% ethanol if 100 µl of Buffer RLT has been used in step 3.

5. Apply the samples (300 µl) from step 4 into the wells of the RNeasy 96 plate, and switch on vacuum source. Apply vacuum until transfer is complete (15–60 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

Make sure that the QIAvac 96 vacuum manifold is assembled correctly before loading. The flow-through is collected in the waste tray.

Note: Tape unused wells with adhesive tape. Do not use the AirPore Tape Sheets supplied with the RNeasy 96 Kit. Use either adhesive tape or Tape Pads (cat. no. 19570) from QIAGEN.

Note: The vacuum must be switched off and the manifold ventilated between pipetting steps to maintain uniform conditions for each sample.

6. DNase digestion (optional)

Note: Generally, DNase digestion is not required since the RNeasy 96 silica-membrane, spin-column technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA. See page 48 for more information and page 18 for details to prepare the DNase I incubation mix.

A. Pipet 80 µl of the DNase I incubation mix (see page 18) directly onto the RNeasy membrane in each well of the RNeasy 96 plate. Seal the plate with an AirPore Tape Sheet.

Note: Make sure to pipet the DNase I incubation mix directly onto the RNeasy membrane. DNase digestion will be incomplete if some of the mix sticks to the walls or the O-rings of the RNeasy 96 plate.

B. Place at room temperature for 15 min.

C. Remove the AirPore Tape from the RNeasy 96 plate. Proceed with step 7.

7. Add 1 ml of Buffer RW1 to each well of the RNeasy 96 plate. (If on-membrane DNase digestion was performed in the previous step, wait 5 min before proceeding.) Switch on vacuum source, and apply vacuum until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

Note: To efficiently remove the DNase following DNase digestion, allow Buffer RW1 to remain on the RNeasy membrane for 5 min before vacuuming the buffer through the membrane.

Collect wash fraction in the same waste tray used in step 5.

8. Lift the top plate carrying the RNeasy 96 plate from the base, and empty the waste tray. Reassemble the QIAvac 96 vacuum manifold.

9. Add 1 ml of Buffer RPE to each well of the RNeasy 96 plate, and switch on vacuum source. Apply vacuum until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

Note: Ensure that ethanol is added to Buffer RPE (see ''Important notes before starting'', page 19).

10. Add another 1 ml of Buffer RPE to each well of the RNeasy 96 plate, and apply vacuum until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

11. Lift the RNeasy 96 plate from the QIAvac top plate, and strike the bottom side of the RNeasy 96 plate on a stack of paper towels (~4 cm high). Repeat several times until no further liquid is released onto the paper towels.

Residual Buffer RPE from the collars and nozzles of each well will be absorbed by the paper towels. Droplets adhering to the nozzles and collars should be removed with a tissue.

12. Place the RNeasy 96 plate back in the QIAvac top plate. Apply vacuum for 10 min. Switch off vacuum, and ventilate QIAvac 96 manifold.

It is important to dry the RNeasy membrane, since residual ethanol may interfere with subsequent reactions. The 10 min vacuum application ensures that no ethanol is carried over during elution.

13. Replace the waste tray with an elution microtube rack containing 1.2 ml elution microtubes assembled on top of the Elution Microtube Adapter.

14. To elute, pipet 60–70 µl of RNase-free water directly onto the membrane in each well. Let stand for 1 min. Then switch on vacuum source until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

Note: Make sure to pipet the RNase-free water directly onto the RNeasy membrane. Elution will be incomplete if some of the water sticks to the walls or the O-rings of the RNeasy 96 plate.

15. Repeat the elution step (step 14) once, as described, with a second volume of 60–70 µl RNase-free water.

Note: Repeating the elution step is required for complete recovery of RNA. The elution volume will be approximately 30 µl less than the volume of RNase-free water added to the membrane, corresponding to the membrane dead volume.

Use caps provided to seal the microtubes for storage. Store RNA at –20°C or at –70°C.

RNeasy 96 Protocol for Isolation of Total RNA from Animal Cells

II. Using vacuum/spin technology

1. Preparation of QIAvac 96 vacuum manifold

Place the waste tray inside the QIAvac base. Place the top plate squarely over the base. Place RNeasy 96 plate in the QIAvac top plate, making sure that the plate is seated tightly. Attach QIAvac 96 manifold to the vacuum source. Keep vacuum switched off.

Note: Always place the RNeasy 96 plate into the vacuum manifold with the beveled edges pointing to the right-hand side.

2. Harvesting cells

a) Cells grown in a monolayer
Cells grown in a monolayer in multiwell cell-culture plates can be lysed directly in the wells. Completely remove medium by pipetting, and continue with step 3.

Note: Incomplete removal of the supernatant will dilute Buffer RLT in subsequent steps, inhibiting lysis and the binding of RNA to the RNeasy membrane. This will lead to reduced yield.

b) Cells grown in suspension
Transfer aliquots of up to 5 x 10^5 cells into a 96-well microplate. Spin cells for 5 min at 300 x *g*, and completely remove supernatant by pipetting. Proceed with step 3.

Note: Incomplete removal of the supernatant will dilute Buffer RLT in subsequent steps, inhibiting lysis and the binding of RNA to the RNeasy membrane. This will lead to reduced yield.

3. Add 150 µl of Buffer RLT to each well of the microplate. Keeping the microplate flat on the bench, shake it vigorously back and forth for 10 s. While continuing to keep the plate flat on the bench, rotate the plate by 90° and shake it for an additional 10 s.

Note: After the addition of ethanol in step 4, total volume will be 300 µl. If the surface of the liquid is too high for convenient use in the 96-well microplate used, the volume of Buffer RLT may be reduced to 100 µl.

4. Add 1 volume (150 µl) of 70% ethanol. Mix by pipetting up and down 3 times.

Note: Add 100 µl of 70% ethanol if 100 µl of Buffer RLT has been used in step 3.

5. Apply the samples (300 µl) from step 4 into the wells of the RNeasy 96 plate, and switch on vacuum source. Apply vacuum until transfer is complete (15–60 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

Make sure QIAvac 96 vacuum manifold is assembled correctly before loading. The flow-through is collected in the waste tray.

Note: Tape unused wells with adhesive tape. Do not use the AirPore Tape Sheets supplied with the RNeasy 96 Kit. Use either adhesive tape or Tape Pads (cat. no. 19570) from QIAGEN.

Note: The vacuum must be switched off and the manifold ventilated between pipetting steps to maintain uniform conditions for each sample.

6. DNase digestion (optional)

Note: Generally, DNase digestion is not required since the RNeasy 96 silica-membrane, spin-column technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA. See page 48 for more information and page 18 for details to prepare the DNase I incubation mix.

A. Pipet 80 µl of the DNase I incubation mix (see page 18) directly onto the RNeasy membrane in each well of the RNeasy 96 plate. Seal the plate with an AirPore Tape Sheet.

Note: Make sure to pipet the DNase I incubation mix directly onto the RNeasy membrane. DNase digestion will be incomplete if some of the mix sticks to the walls or the O-rings of the RNeasy 96 plate.

B. Place at room temperature for 15 min.

C. Remove the AirPore Tape from the RNeasy 96 plate. Proceed with step 7.

7. Add 1 ml of Buffer RW1 to each well of the RNeasy 96 plate. (If on-membrane DNase digestion was performed in the previous step, wait 5 min before proceeding.) Switch on vacuum source, and apply vacuum until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

Note: To efficiently remove the DNase following DNase digestion, allow Buffer RW1 to remain on the RNeasy membrane for 5 min before vacuuming the buffer through the membrane.

Collect wash fraction in the same waste tray used in step 5.

8. Lift the top plate carrying the RNeasy 96 plate from the base, and empty the waste tray. Reassemble the QIAvac 96 vacuum manifold.

9. Add 1 ml of Buffer RPE to each well of the RNeasy 96 plate, and switch on vacuum source. Apply vacuum until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

Note: Ensure that ethanol is added to Buffer RPE (see ''Important notes before starting'', page 19).

10. Place the RNeasy 96 plate on top of a Square-Well Block. Mark the RNeasy plate for later identification.

11. Add another 1 ml of Buffer RPE to each well of the RNeasy 96 plate. Seal the RNeasy 96 plate with an AirPore Tape Sheet. Load the Square-Well Block and RNeasy 96 plate into the holder, and place the whole assembly in the rotor bucket. Centrifuge at 6000 rpm (~5600 x *g*) for 10 min at room temperature to dry the plate membranes.

Centrifugation with sealed plates prevents cross-contamination.

It is important to dry the RNeasy membrane since residual ethanol may interfere with subsequent reactions. The 10 min spin ensures that residual traces of salt are removed and that no ethanol is carried over during elution.

12. Remove the AirPore Tape. Place RNeasy 96 plate on top of an elution microtube rack containing 1.2 ml elution microtubes.

13. To elute the RNA, add 45–70 µl of RNase-free water to each well, and seal the RNeasy 96 plate with a new sheet of AirPore Tape. Incubate for 1 min at room temperature. Then centrifuge at 6000 rpm (~5600 x *g*) for 4 min at room temperature.

Note: Make sure to pipet the RNase-free water directly onto the RNeasy membrane. Elution will be incomplete if some of the water sticks to the walls or the O-rings of the RNeasy 96 plate.

14. Remove the AirPore Tape. Repeat the elution step (step 13) once with a second volume of 45–70 µl RNase-free water.

Note: Repeating the elution step is required for complete recovery of RNA. The elution volume will be approximately 15 µl less than the volume of RNase-free water added to the membrane, corresponding to the membrane dead volume.

Use caps provided to seal the microtubes for storage. Store RNA at –20°C or at –70°C.

RNeasy 96 Protocol for Isolation of Total RNA from Animal Cells

III.Using spin technology

1. Harvesting cells

a) Cells grown in a monolayer
Cells grown in a monolayer in multiwell cell-culture plates can be lysed directly in the wells. Completely remove medium by pipetting, and continue with step 2.

Note: Incomplete removal of the supernatant will dilute Buffer RLT in subsequent steps, inhibiting lysis and the binding of RNA to the RNeasy membrane. This will lead to reduced yield.

b) Cells grown in suspension
Transfer aliquots of up to 5 x 10^5 cells into a 96-well microplate. Spin cells for 5 min at 300 x *g*, and completely remove supernatant by pipetting. Proceed with step 2.

Note: Incomplete removal of the supernatant will dilute Buffer RLT in subsequent steps, inhibiting lysis and the binding of RNA to the RNeasy membrane. This will lead to reduced yield.

2. Add 150 µl of Buffer RLT to each well of the microplate. Keeping the microplate flat on the bench, shake it vigorously back and forth for 10 s. While continuing to keep the plate flat on the bench, rotate the plate by 90° and shake it for an additional 10 s.

Note: After the addition of ethanol in step 4, total volume will be 300 µl. If the surface of the liquid is too high for convenient use in the 96-well microplate used, the volume of Buffer RLT may be reduced to 100 µl.

3. Add 1 volume (150 µl) of 70% ethanol to each microplate well. Mix by pipetting up and down 3 times.

Note: Add 100 µl of 70% ethanol if 100 µl of Buffer RLT has been used in step 2.

4. Place an RNeasy 96 plate on top of a Square-Well Block. Mark the RNeasy 96 plate for later identification.

5. Apply the samples from step 3 into the wells of the RNeasy 96 plate.

Take care not to wet the rims of the wells to avoid cross-contamination in subsequent steps.

6. Seal the RNeasy 96 plate with an AirPore Tape Sheet. Load the Square-Well Block and RNeasy 96 plate into the holder, and place the whole assembly in the rotor bucket. Centrifuge at 6000 rpm (~5600 x g) for 4 min at room temperature.

Centrifugation with sealed plates prevents cross-contamination.

7. DNase digestion (optional)

Note: Generally, DNase digestion is not required since the RNeasy 96 silica-membrane, spin-column technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA. See page 48 for more information and page 18 for details to prepare the DNase I incubation mix.

A. Remove the AirPore Tape. Pipet 80 µl of the DNase I incubation mix (see page 18) directly onto the RNeasy membrane in each well of the RNeasy 96 plate. Seal the plate with a new sheet of AirPore Tape.

Note: Make sure to pipet the DNase I incubation mix directly onto the RNeasy membrane. DNase digestion will be incomplete if some of the mix sticks to the walls or the O-rings of the RNeasy 96 plate.

B. Place at room temperature for 15 min. Then proceed with step 8.

8. **Remove AirPore Tape. Add 0.8 ml of Buffer RW1 to each well of the RNeasy 96 plate. Seal the RNeasy 96 plate with a new sheet of AirPore Tape. (If on-membrane DNase digestion was performed in the previous step, wait 5 min before proceeding.) Centrifuge at 6000 rpm (~5600 x *g*) for 4 min at room temperature.**

   Note: To efficiently remove the DNase following DNase digestion, allow Buffer RW1 to remain on the RNeasy membrane for 5 min before centrifuging the buffer through the membrane.

9. **Place the RNeasy 96 plate on top of another clean Square-Well Block. Remove AirPore Tape. Add 0.8 ml of Buffer RPE to each well of the RNeasy 96 plate. Seal the RNeasy 96 plate with a new sheet of AirPore Tape. Centrifuge at 6000 rpm (~5600 x *g*) for 4 min at room temperature.**

   Note: Ensure that ethanol is added to Buffer RPE (see "Important notes before starting", page 19).

10. **Remove AirPore Tape. Add another 0.8 ml of Buffer RPE to each well of the RNeasy 96 plate. Seal the RNeasy 96 plate with a new piece of AirPore Tape. Centrifuge at 6000 rpm (~5600 x *g*) for 10 min at room temperature.**

    It is important to dry the RNeasy membrane since residual ethanol may interfere with subsequent reactions. The 10 min spin ensures that residual traces of salt are removed and that no ethanol is carried over during elution.

11. **Remove AirPore Tape. Place RNeasy 96 plate on top of an elution microtube rack containing 1.2 ml elution microtubes.**

12. **To elute the RNA, add 45–70 µl of RNase-free water to each well, and seal the RNeasy 96 plate with a new piece of AirPore Tape. Incubate for 1 min at room temperature. Then centrifuge at 6000 rpm (~5600 x *g*) for 4 min at room temperature.**

    Note: Make sure to pipet the RNase-free water directly onto the RNeasy membrane. Elution will be incomplete if some of the water sticks to the walls or the O-rings of the RNeasy 96 plate.

13. **Remove AirPore Tape. Repeat the elution step (step 12) once with a second volume of 45–70 µl RNase-free water.**

    Note: Repeating the elution step is required for complete recovery of RNA. The elution volume will be approximately 15 µl less than the volume of RNase-free water added to the membrane, corresponding to the membrane dead volume.

    Use caps provided to seal the microtubes for storage. Store RNA at –20°C or at –70°C.

RNeasy 96 Protocol for Isolation of Cytoplasmic RNA from Animal Cells

Important notes before starting

- If preparing RNA for the first time, please read Appendix A (page 45). If using the RNeasy 96 Kit for the first time, please read ''Important Notes before Using the RNeasy 96 Kit'' (page 12).
- All centrifugation steps in the vacuum/spin protocol and in the spin protocol are performed in a Centrifuge 4-15C or Centrifuge 4K15C. Pelleting of nuclei can also be performed in a standard 96-well–microplate centrifuge. However, if nuclei from RNase-rich cells are pelleted at 4°C to avoid degradation of RNA, a refrigerated 96-well–microplate centrifuge or Centrifuge 4K15C, the refrigerated version of Centrifuge 4-15C, is required (see page 16 and protocols).
- Use of a multichannel pipet is recommended (see page 17). Pour buffers and RNase-free water into reagent reservoirs for multichannel pipets. Use reservoirs from a freshly opened package or clean them as described for Square-Well Blocks (see page 17).
- A vacuum source capable of generating a vacuum pressure of –800 to –900 mbar is necessary for the vacuum and vacuum/spin protocol (see page 15). The vacuum must be switched off and the manifold ventilated between pipetting steps to maintain uniform conditions for each sample.
- Only use freshly harvested cells since ice crystals form during freezing and thawing and destroy the nuclear membranes, releasing DNA and other nuclear molecules.
- When isolating RNA from cells containing high amounts of RNases, in some cases it may be necessary to add β-mercaptoethanol (β-ME) to Buffer RLT and RNase inhibitor and DTT to Buffer RLN to avoid degradation of RNA. β-ME supports the inactivation of RNases by GITC. Add 10 µl of 14.5 M β-ME per 1 ml of Buffer RLT. Buffer RLT is stable for 1 month after addition of β-ME. Add 1000 U/ml RNase inhibitor and 1 mM DTT to Buffer RLN just before use. In most cases, it is not necessary to add β-ME to Buffer RLT or an RNase inhibitor to Buffer RLN.
- Cell lysis is performed on ice. All subsequent steps of the RNeasy protocol should be performed at room temperature (20 to 30°C). Avoid interruptions during the procedure.
- Chill Buffer RLN in a refrigerator to 2–8°C or on ice. With the exception of Buffer RLN, buffers should not be precooled.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) to obtain a working solution.
- Buffer RLT may form a precipitate upon storage. If necessary, warm to 37°C to redissolve.
- Using the cytoplasmic protocol, DNase digestion is generally not required: most of the DNA is removed by pelleting the nuclei during the procedure, and the RNeasy 96 silica-membrane technology efficiently removes nearly all of the remaining small amounts of DNA without DNase treatment. However, further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA. See page 48 for more information and page 18 for details to prepare the DNase I incubation mix.

RNeasy 96 Protocol for Isolation of Cytoplasmic RNA from Animal Cells

I. Using vacuum technology

1. Preparation of QIAvac 96 vacuum manifold

Place the waste tray inside the QIAvac base. Place the top plate squarely over the base. Place the RNeasy 96 plate in the QIAvac top plate, making sure that the plate is seated tightly. Attach QIAvac 96 manifold to the vacuum source. Keep vacuum switched off.

Note: Always place the RNeasy 96 plate into the vacuum manifold with the beveled edges pointing to the right-hand side.

2. Harvesting cells

a) Cells grown in a monolayer
Cells grown in a monolayer in multiwell cell-culture plates can be lysed directly in the wells. Completely remove medium by pipetting using a multichannel pipet, and continue with step 3.

Note: Incomplete removal of the supernatant will dilute Buffers RLN and RLT in subsequent steps, inhibiting lysis and the binding of RNA to the RNeasy membrane. This will lead to reduced yield.

b) Cells grown in suspension
Transfer aliquots of up to 5 x 10^5 cells into a 96-well microplate. Spin cells for 5 min at 300 x *g*, and completely remove supernatant with a multichannel pipet. Proceed with step 3.

Note: Incomplete removal of the supernatant will dilute Buffers RLN and RLT in subsequent steps, inhibiting lysis and the binding of RNA to the RNeasy membrane. This will lead to reduced yield.

3. Add 100 µl of ice-cold Buffer RLN to each microplate well. Keeping the microplate flat on the bench, shake it vigorously back and forth for 10 s. While continuing to keep the plate flat on the bench, rotate the plate by 90° and shake it for an additional 10 s. Incubate for 5 min on ice, keeping the microplate covered.

Note: At this step, cell lysates have to be processed immediately and cannot be stored for later use.

4. Centrifuge the microplate for 5 min at 1500 rpm (300–500 x *g*) to pellet the cell nuclei. During centrifugation, proceed with step 5.

Note: For most preparations, centrifugation can be performed at room temperature. In some cases, when analyzing transcripts from RNase-rich cells, it may be advantageous to perform the centrifugation at 4°C.

5. Pipet 350 µl of room-temperature Buffer RLT into each well of a Square-Well Block.

6. Transfer supernatants from step 4 into the Square-Well Block containing Buffer RLT, and mix by pipetting up and down 3 times.

To transfer the supernatants, hold the microplate at an angle from horizontal, and position the pipet tip at the edge of the well bottom to minimize the risk of carrying over pelleted cell nuclei.

7. Add 250 µl of 96–100% ethanol, and mix again by pipetting up and down 3 times.

8. **Apply the samples (700 µl) from step 7 into the wells of the RNeasy 96 plate, and switch on vacuum source. Apply vacuum until transfer is complete (15–60 s). Switch off vacuum, and ventilate QIAvac 96 manifold.**

   Make sure QIAvac 96 vacuum manifold is assembled correctly before loading. The flow-through is collected in the waste tray.

   Note: Tape unused wells with adhesive tape. Do not use the AirPore Tape Sheets supplied with the RNeasy 96 Kit. Use either adhesive tape or Tape Pads (cat. no. 19570) from QIAGEN.

   Note: The vacuum must be switched off and the manifold ventilated between pipetting steps to maintain uniform conditions for each sample.

9. **DNase digestion (optional)**

   Note: Using the cytoplasmic protocol, DNase digestion is generally not required: most of the DNA is removed by pelleting the nuclei in step 4, and the RNeasy 96 silica-membrane technology efficiently removes nearly all of the remaining small amounts of DNA without DNase treatment. However, further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA. Using the cytoplasmic protocol with the optional DNase digestion results in undetectable levels of DNA, even by sensitive TaqMan analysis. See page 48 for more information and page 18 for details to prepare the DNase I incubation mix.

   A. Pipet 80 µl of the DNase I incubation mix (see page 18) directly onto the RNeasy membrane in each well of the RNeasy 96 plate. Seal the plate with AirPore Tape.

      Note: Make sure to pipet the DNase I incubation mix directly onto the RNeasy membrane. DNase digestion will be incomplete if some of the mix sticks to the walls or the O-rings of the RNeasy 96 plate.

   B. Place at room temperature for 15 min.

   C. Remove AirPore Tape from the RNeasy 96 plate. Proceed with step 10.

10. **Add 1 ml of Buffer RW1 to each well of the RNeasy 96 plate. (If on-membrane DNase digestion was performed in the previous step, wait 5 min before proceeding.) Switch on vacuum source, and apply vacuum until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.**

    Note: To efficiently remove the DNase following DNase digestion, allow Buffer RW1 to remain on the RNeasy membrane for 5 min before vacuuming the buffer through the membrane.

    Collect wash fraction in the same waste tray used in step 8.

11. **Lift the top plate carrying the RNeasy 96 plate from the base, and empty the waste tray. Reassemble the QIAvac 96 vacuum manifold.**

12. **Add 1 ml of Buffer RPE to each well of the RNeasy 96 plate, and switch on vacuum source. Apply vacuum until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.**

    Note: Ensure that ethanol is added to Buffer RPE (see ''Important notes before starting'', page 26).

13. **Add another 1 ml of Buffer RPE to each well of the RNeasy 96 plate, and apply vacuum until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.**

14. **Lift the RNeasy 96 plate from the QIAvac top plate, and strike the bottom side of the RNeasy 96 plate on a stack of paper towels (~4 cm high). Repeat several times until no further liquid is released onto the paper towels.**

    Residual Buffer RPE from the collars and nozzles of each well will be absorbed by the paper towels. Droplets adhering to the nozzles and collars should be removed with a tissue.

15. **Place the RNeasy 96 plate back in the QIAvac top plate. Apply vacuum for 10 min. Switch off vacuum, and ventilate QIAvac 96 manifold.**

    It is important to dry the RNeasy membrane, since residual ethanol may interfere with subsequent reactions. The 10 min vacuum application ensures that no ethanol is carried over during elution.

16. **Replace the waste tray with an elution microtube rack containing 1.2 ml elution microtubes assembled on top of the Elution Microtube Adapter.**

17. **To elute, pipet 60–70 µl of RNase-free water directly onto the membrane in each well. Let stand for 1 min. Then switch on vacuum source until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.**

    Note: Make sure to pipet the RNase-free water directly onto the RNeasy membrane. Elution will be incomplete if some of the water sticks to the walls or the O-rings of the RNeasy 96 plate.

18. **Repeat the elution step (step 17) once, as described, with a second volume of 60–70 µl RNase-free water.**

    Note: Repeating the elution step is required for complete recovery of RNA. The elution volume will be 30 µl less than the volume of RNase-free water added to the membrane, corresponding to the membrane dead volume.

    Use caps provided to seal the microtubes for storage. Store RNA at –20°C or at –70°C.

RNeasy 96 Protocol for Isolation of Cytoplasmic RNA from Animal Cells

II. Using vacuum/spin technology

1. **Preparation of QIAvac 96 vacuum manifold**

   Place the waste tray inside the QIAvac base. Place the top plate squarely over the base. Place RNeasy 96 plate in the QIAvac top plate, making sure that the plate is seated tightly. Attach QIAvac 96 manifold to the vacuum source. Keep vacuum switched off.

   Note: Always place the RNeasy 96 plate into the vacuum manifold with the beveled edges pointing to the right-hand side.

2. **Harvesting cells**

   a) **Cells grown in a monolayer**
   Cells grown in a monolayer in multiwell cell-culture plates can be lysed directly in the wells. Completely remove medium with a multichannel pipet, and continue with step 3.

   Note: Incomplete removal of the supernatant will dilute Buffers RLN and RLT in subsequent steps, inhibiting lysis and the binding of RNA to the RNeasy membrane. This will lead to reduced yield.

   b) **Cells grown in suspension**
   Transfer aliquots of up to 5 x 10^5 cells into a 96-well microplate. Spin cells for 5 min at 300 x *g*, and completely remove supernatant with a multichannel pipet. Proceed with step 3.

   Note: Incomplete removal of the supernatant will dilute Buffers RLN and RLT in subsequent steps, inhibiting lysis and the binding of RNA to the RNeasy membrane. This will lead to reduced yield.

3. **Add 100 µl of ice-cold Buffer RLN to each microplate well. Keeping the microplate flat on the bench, shake it vigorously back and forth for 10 s. While continuing to keep the plate flat on the bench, rotate the plate by 90° and shake it for an additional 10 s. Incubate for 5 min on ice, keeping the microplate covered.**

   Note: At this step, cell lysates have to be processed immediately and cannot be stored for later use.

4. **Centrifuge the microplate for 5 min at 1500 rpm (300–500 x *g*) to pellet the cell nuclei. During centrifugation, proceed with step 5.**

   Note: For most preparations, centrifugation can be performed at room temperature. In some cases, when analyzing transcripts from RNase-rich cells or when analyzing labile transcripts, it may be advantageous to perform the centrifugation at 4°C.

5. **Pipet 350 µl of room-temperature Buffer RLT into each well of a Square-Well Block.**

6. **Transfer supernatants from step 4 into the Square-Well Block containing Buffer RLT, and mix by pipetting up and down 3 times.**

   To transfer the supernatants, hold the microplate at an angle from horizontal, and position the pipet tip at the edge of the well bottom to minimize the risk of carrying over pelleted cell nuclei.

7. **Add 250 µl of 96–100% ethanol, and mix again by pipetting up and down 3 times.**

8. Apply the samples (700 µl) from step 7 into the wells of the RNeasy 96 plate, and switch on vacuum source. Apply vacuum until transfer is complete (15–60 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

Make sure QIAvac 96 vacuum manifold is assembled correctly before loading. The flow-through is collected in the waste tray.

Note: Tape unused wells with adhesive tape. Do not use the AirPore Tape Sheets supplied with the RNeasy 96 Kit. Use either adhesive tape or Tape Pads (cat. no. 19570) from QIAGEN.

Note: The vacuum must be switched off and the manifold ventilated between pipetting steps to maintain uniform conditions for each sample.

9. DNase digestion (optional)

Note: Using the cytoplasmic protocol, DNase digestion is generally not required: most of the DNA is removed by pelleting the nuclei in step 4, and the RNeasy 96 silica-membrane technology efficiently removes nearly all of the remaining small amounts of DNA without DNase treatment. However, further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA. Using the cytoplasmic protocol with the optional DNase digestion results in undetectable levels of DNA, even by sensitive TaqMan analysis. See page 48 for more information and page 18 for details to prepare the DNase I incubation mix.

A. Pipet 80 µl of the DNase I incubation mix (see page 18) directly onto the RNeasy membrane in each well of the RNeasy 96 plate. Seal the plate with AirPore Tape.

Note: Make sure to pipet the DNase I incubation mix directly onto the RNeasy membrane. DNase digestion will be incomplete if some of the mix sticks to the walls or the O-rings of the RNeasy 96 plate.

B. Place at room temperature for 15 min.

C. Remove AirPore Tape from the RNeasy 96 plate. Proceed with step 10.

10. Add 1 ml of Buffer RW1 to each well of the RNeasy 96 plate. (If on-membrane DNase digestion was performed in the previous step, wait 5 min before proceeding.) Switch on vacuum source, and apply vacuum until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

Note: To efficiently remove the DNase following DNase digestion, allow Buffer RW1 to remain on the RNeasy membrane for 5 min before vacuuming the buffer through the membrane.

Collect wash fraction in the same waste tray used in step 8.

11. Lift the top plate carrying the RNeasy 96 plate from the base, and empty the waste tray. Reassemble the QIAvac 96 vacuum manifold.

12. Add 1 ml of Buffer RPE to each well of the RNeasy 96 plate, and switch on vacuum source. Apply vacuum until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

Note: Ensure that ethanol is added to Buffer RPE (see ''Important notes before starting'', page 26).

13. Place the RNeasy 96 plate on top of a Square-Well Block. Mark the RNeasy plate for later identification.

14. **Add another 1 ml of Buffer RPE to each well of the RNeasy 96 plate. Seal the RNeasy 96 plate with AirPore Tape. Load the Square-Well Block and RNeasy 96 plate into the holder, and place the whole assembly in the rotor bucket. Centrifuge at 6000 rpm (~5600 x *g*) for 10 min at room temperature to dry the plate membranes.**

    Centrifugation with sealed plates prevents cross-contamination.

    It is important to dry the RNeasy membrane since residual ethanol may interfere with subsequent reactions. The 10 min spin ensures that residual traces of salt are removed and that no ethanol is carried over during elution.

15. **Remove AirPore Tape. Place RNeasy 96 plate on top of an elution microtube rack containing 1.2 ml elution microtubes.**

16. **To elute the RNA, add 45–70 µl of RNase-free water to each well, and seal the RNeasy 96 plate with a new sheet of AirPore Tape. Incubate for 1 min at room temperature. Then centrifuge at 6000 rpm (~5600 x *g*) for 4 min at room temperature.**

    Note: Make sure to pipet the RNase-free water directly onto the RNeasy membrane. Elution will be incomplete if some of the water sticks to the walls or the O-rings of the RNeasy 96 plate.

17. **Remove AirPore Tape. Repeat the elution step (step 16) once with a second volume of 45–70 µl RNase-free water.**

    Note: Repeating the elution step is required for complete recovery of RNA. The elution volume will be 15 µl less than the volume of RNase-free water added to the membrane, corresponding to the membrane dead volume.

    Use caps provided to seal the microtubes for storage. Store RNA at –20°C or at –70°C.

RNeasy 96 Protocol for Isolation of Cytoplasmic RNA from Animal Cells

III. Using spin technology

1. **Harvesting cells**

   a) **Cells grown in a monolayer**
   Cells grown in a monolayer in multiwell cell-culture plates can be lysed directly in the wells. Completely remove medium with a multichannel pipet, and continue with step 3.

   Note: Incomplete removal of the supernatant will dilute Buffers RLN and RLT in subsequent steps, inhibiting lysis and the binding of RNA to the RNeasy membrane. This will lead to reduced yield.

   b) **Cells grown in suspension**
   Transfer aliquots of up to 5 x 10^5 cells into a 96-well microplate. Spin cells for 5 min at 300 x *g*, and completely remove supernatant with a multichannel pipet. Proceed with step 3.

   Note: Incomplete removal of the supernatant will dilute Buffers RLN and RLT in subsequent steps, inhibiting lysis and the binding of RNA to the RNeasy membrane. This will lead to reduced yield.

2. **Add 100 µl of ice-cold Buffer RLN to each microplate well. Keeping the microplate flat on the bench, shake it vigorously back and forth for 10 s. While continuing to keep the plate flat on the bench, rotate the plate by 90° and shake it for an additional 10 s. Incubate for 5 min on ice, keeping the microplate covered.**

   Note: At this step, cell lysates have to be processed immediately and cannot be stored for later use.

3. **Centrifuge the microplate for 5 min at 1500 rpm (300–500 x *g*) to pellet the cell nuclei. During centrifugation, proceed with step 4.**

   Note: For most preparations, centrifugation can be performed at room temperature. In some cases, when analyzing transcripts from RNase-rich cells or when analyzing labile transcripts, it may be advantageous to perform the centrifugation at 4°C.

4. **Pipet 350 µl of room-temperature Buffer RLT into each well of a Square-Well Block.**

5. **Transfer supernatants from step 3 into the Square-Well Block containing Buffer RLT, and mix by pipetting up and down 3 times.**

   To transfer the supernatants, hold the microplate at an angle from horizontal, and position the pipet tip at the edge of the well bottom to minimize the risk of carrying over pelleted cell nuclei.

6. **Add 250 µl of 96–100% ethanol, and mix again by pipetting up and down 3 times.**

7. **Place an RNeasy 96 plate on top of a Square-Well Block. Mark the RNeasy 96 plate for later identification.**

8. **Apply the samples from step 6 (700 µl) into the wells of the RNeasy 96 plate.**

   Take care not to wet the rims of the wells to avoid cross-contamination in subsequent steps.

9. **Seal the RNeasy 96 plate with AirPore Tape. Load the Square-Well Block and RNeasy 96 plate into the holder, and place the entire assembly in the rotor bucket. Centrifuge at 6000 rpm (~5600 x *g*) for 4 min at room temperature.**

   Centrifugation with sealed plates prevents cross-contamination.

10. DNase digestion (optional)

Note: Using the cytoplasmic protocol, DNase digestion is generally not required: most of the DNA is removed by pelleting the nuclei in step 3, and the RNeasy 96 silica-membrane technology efficiently removes nearly all of the remaining small amounts of DNA without DNase treatment. However, further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA. Using the cytoplasmic protocol with the optional DNase digestion results in undetectable levels of DNA, even by sensitive TaqMan analysis. See page 48 for more information and page 18 for details to prepare the DNase I incubation mix.

A. Remove AirPore Tape. Pipet 80 µl of the DNase I incubation mix (see page 18) directly onto the RNeasy membrane in each well of the RNeasy 96 plate. Seal the plate with a new sheet of AirPore Tape.

Note: Make sure to pipet the DNase I incubation mix directly onto the RNeasy membrane. DNase digestion will be incomplete if some of the mix sticks to the walls or the O-rings of the RNeasy 96 plate.

B. Place at room temperature for 15 min. Then proceed with step 11.

11. Remove AirPore Tape. Add 0.8 ml of Buffer RW1 to each well of the RNeasy 96 plate. Seal the RNeasy 96 plate with a new sheet of AirPore Tape. (If on-membrane DNase digestion was performed in the previous step, wait 5 min before proceeding.) Centrifuge at 6000 rpm (~5600 x *g*) for 4 min at room temperature.

Note: To efficiently remove the DNase following DNase digestion, allow Buffer RW1 to remain on the RNeasy membrane for 5 min before centrifuging the buffer through the membrane.

12. Place the RNeasy 96 plate on top of a another clean Square-Well Block. Remove AirPore Tape. Add 0.8 ml of Buffer RPE to each well of the RNeasy 96 plate. Seal the RNeasy 96 plate with a new sheet of AirPore Tape. Centrifuge at 6000 rpm (~5600 x *g*) for 4 min at room temperature.

Note: Ensure that ethanol is added to Buffer RPE (see "Important notes before starting", page 26).

13. Remove AirPore Tape. Add another 0.8 ml of Buffer RPE to each well of the RNeasy 96 plate. Seal the RNeasy 96 plate with a new sheet of AirPore Tape. Centrifuge at 6000 rpm (~5600 x *g*) for 10 min at room temperature.

It is important to dry the RNeasy membrane since residual ethanol may interfere with subsequent reactions. The 10 min spin ensures that residual traces of salt are removed and that no ethanol is carried over during elution.

14. Remove AirPore Tape. Place RNeasy 96 plate on top of an elution microtube rack containing 1.2 ml elution microtubes.

15. To elute the RNA, add 45–70 µl of RNase-free water to each well, and seal the RNeasy 96 plate with a new sheet of AirPore Tape. Incubate for 1 min at room temperature. Then centrifuge at 6000 rpm (~5600 x *g*) for 4 min at room temperature.

Note: Make sure to pipet the RNase-free water directly onto the RNeasy membrane. Elution will be incomplete if some of the water sticks to the walls or the O-rings of the RNeasy 96 plate.

16. Remove AirPore Tape. Repeat the elution step (step 15) once with a second volume of 45–70 µl RNase-free water.

Note: Repeating the elution step is required for complete recovery of RNA. The elution volume will be 15 µl less than the volume of RNase-free water added to the membrane, corresponding to the membrane dead volume.

Use caps provided to seal the microtubes for storage. Store RNA at –20°C or at –70°C.

RNeasy 96 Protocol for RNA Cleanup

Important notes before starting

- If preparing RNA for the first time, please read Appendix A (page 45). If using the RNeasy 96 Kit for the first time, please read ''Important Notes before Using the RNeasy 96 Kit'' (page 12).
- All centrifugation steps in the vacuum/spin protocol and in the spin protocol are performed in a Centrifuge 4-15C or Centrifuge 4K15C (see page 16).
- Use of a multichannel pipet is recommended (see page 17). Pour Buffers and RNase-free water into reagent reservoirs for multichannel pipets. Use reservoirs from a freshly opened package or clean them as described for Square-Well Blocks (see page 17).
- A vacuum source capable of generating a vacuum pressure of –800 to –900 mbar is necessary for the vacuum and vacuum/spin protocol (see page 15). The vacuum must be switched off and the manifold ventilated between pipetting steps to maintain uniform conditions for each sample.
- Buffer RLT may form a precipitate upon storage. If necessary, warm to redissolve.
- Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) to obtain a working solution.
- All steps of the RNeasy 96 cleanup protocol should be performed at room temperature (20 to 30°C). Avoid interruptions during the procedure.
- Generally, DNase digestion is not required since the RNeasy 96 silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA. We recommend using the optional on-column DNase digestion step or a DNase digestion of the reaction mixture before starting the procedure. See page 48 for more information and page 18 for details to prepare the DNase I incubation mix.

RNeasy 96 Protocol for RNA Cleanup

I. Using vacuum technology

1. Preparation of QIAvac 96 vacuum manifold

Place the waste tray inside the QIAvac base. Place the top plate squarely over the base. Place the RNeasy 96 plate in the QIAvac top plate, making sure that the plate is seated tightly. Attach QIAvac 96 manifold to the vacuum source. Keep vacuum switched off.

Note: Always place the RNeasy 96 plate into the vacuum manifold with the beveled edges pointing to the right-hand side.

2. Adjust each sample volume to 100 µl with RNase-free water. Add 350 µl Buffer RLT to each sample, and mix by pipetting up and down 3 times with a multichannel pipet.

3. Add 250 µl of ethanol (96–100%) to each sample, and mix by pipetting up and down 3 times with a multichannel pipet.

4. Apply the samples from step 3 (700 µl) into the wells of the RNeasy 96 plate, and switch on vacuum source. Apply vacuum until transfer is complete (15–60 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

Make sure QIAvac 96 vacuum manifold is assembled correctly before loading. The flow-through is collected in the waste tray.

Note: Tape unused wells with adhesive tape. Do not use the AirPore Tape Sheets supplied with the RNeasy 96 Kit. Use either adhesive tape or Tape Pads (cat. no. 19570) from QIAGEN.

Note: The vacuum must be switched off and the manifold ventilated between pipetting steps to maintain uniform conditions for each sample.

5. DNase digestion (optional)

Note: Generally, DNase digestion is not required since the RNeasy 96 silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA. See page 48 for more information and page 18 for details to prepare the DNase I incubation mix.

A. Pipet 80 µl of the DNase I incubation mix (see page 18) directly onto the RNeasy membrane in each well of the RNeasy 96 plate. Seal the plate with AirPore Tape.

Note: Make sure to pipet the DNase I incubation mix directly onto the RNeasy membrane. DNase digestion will be incomplete if some of the mix sticks to the walls or the O-rings of the RNeasy 96 plate.

B. Place at room temperature for 15 min.

C. Remove AirPore Tape from the RNeasy 96 plate. Proceed with step 6.

6. Add 1 ml of Buffer RW1 to each well of the RNeasy 96 plate. (If on-membrane DNase digestion was performed in the previous step, wait 5 min before proceeding.) Switch on vacuum source, and apply vacuum until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

Note: To efficiently remove the DNase following DNase digestion, allow Buffer RW1 to remain on the RNeasy membrane for 5 min before vacuuming the buffer through the membrane.

Collect wash fraction in the same waste tray used in step 4.

7. Lift the top plate carrying the RNeasy 96 plate from the base, and empty the waste tray. Reassemble the QIAvac 96 vacuum manifold.

8. **Add 1 ml of Buffer RPE to each well of the RNeasy 96 plate, and switch on vacuum source. Apply vacuum until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.**

   Note: Ensure that ethanol is added to Buffer RPE (see ''Important notes before starting'', page 35).

9. **Add another 1 ml of Buffer RPE to each well of the RNeasy 96 plate, and apply vacuum until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.**

10. **Lift the RNeasy 96 plate from the QIAvac top plate, and strike the bottom side of the RNeasy 96 plate on a stack of paper towels (~4 cm high). Repeat several times until no further liquid is released onto the paper towels.**

    Residual Buffer RPE from the collars and nozzles of each well will be absorbed by the paper towels. Droplets adhering to the nozzles and collars should be removed with a tissue.

11. **Place the RNeasy 96 plate back in the QIAvac top plate. Apply vacuum for 10 min. Switch off vacuum, and ventilate QIAvac 96 manifold.**

    It is important to dry the RNeasy membrane, since residual ethanol may interfere with subsequent reactions. The 10 min vacuum application ensures that no ethanol is carried over during elution.

12. **Replace the waste tray with an elution microtube rack containing 1.2 ml elution microtubes assembled on top of the Elution Microtube Adapter.**

13. **To elute, pipet 60–70 µl of RNase-free water directly onto the membrane in each well. Let stand for 1 min. Then switch on vacuum source until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.**

    Note: Make sure to pipet the RNase-free water directly onto the RNeasy membrane. Elution will be incomplete if some of the water sticks to the walls or the O-rings of the RNeasy 96 plate.

14. **Repeat the elution step (step 13) once, as described, with a second volume of 60–70 µl RNase-free water.**

    Note: Repeating the elution step is required for complete recovery of RNA. The elution volume will be 30 µl less than the volume of RNase-free water added to the membrane, corresponding to the membrane dead volume.

    Use caps provided to seal the microtubes for storage. Store RNA at –20°C or at –70°C.

RNeasy 96 Protocol for RNA Cleanup

II. Using vacuum/spin technology

1. Preparation of QIAvac 96 vacuum manifold

Place the waste tray inside the QIAvac base. Place the top plate squarely over the base. Place RNeasy 96 plate in the QIAvac top plate, making sure that the plate is seated tightly. Attach QIAvac 96 manifold to the vacuum source. Keep vacuum switched off.

Note: Always place the RNeasy 96 plate into the vacuum manifold with the beveled edges pointing to the right-hand side.

2. Adjust each sample volume to 100 µl with RNase-free water. Add 350 µl Buffer RLT to each sample, and mix by pipetting up and down 3 times with a multichannel pipet.

3. Add 250 µl of ethanol (96–100%) to each sample, and mix by pipetting up and down 3 times with a multichannel pipet.

4. Apply the samples from step 3 (700 µl) into the wells of the RNeasy 96 plate, and switch on vacuum source. Apply vacuum until transfer is complete (15–60 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

Make sure QIAvac 96 vacuum manifold is assembled correctly before loading. The flow-through is collected in the waste tray.

Note: Tape unused wells with adhesive tape. Do not use the AirPore Tape Sheets supplied with the RNeasy 96 Kit. Use either adhesive tape or Tape Pads (cat. no. 19570) from QIAGEN.

Note: The vacuum must be switched off and the manifold ventilated between pipetting steps to maintain uniform conditions for each sample.

5. DNase digestion (optional)

Note: Generally, DNase digestion is not required since the RNeasy 96 silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA. See page 48 for more information and page 18 for details to prepare the DNase I incubation mix.

A. Pipet 80 µl of the DNase I incubation mix (see page 18) directly onto the RNeasy membrane in each well of the RNeasy 96 plate. Seal the plate with AirPore Tape.

Note: Make sure to pipet the DNase I incubation mix directly onto the RNeasy membrane. DNase digestion will be incomplete if some of the mix sticks to the walls or the O-rings of the RNeasy 96 plate.

B. Place at room temperature for 15 min.

C. Remove AirPore Tape from the RNeasy 96 plate. Proceed with step 6.

6. Add 1 ml of Buffer RW1 to each well of the RNeasy 96 plate. (If on-membrane DNase digestion was performed in the previous step, wait 5 min before proceeding.) Switch on vacuum source, and apply vacuum until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.

Note: To efficiently remove the DNase following DNase digestion, allow Buffer RW1 to remain on the RNeasy membrane for 5 min before vacuuming the buffer through the membrane.

Collect wash fraction in the same waste tray used in step 4.

7. Lift the top plate holding the RNeasy 96 plate from the base, and empty the waste tray. Reassemble the QIAvac 96 vacuum manifold.

8. **Add 1 ml of Buffer RPE to each well of the RNeasy 96 plate, and switch on vacuum source. Apply vacuum until transfer is complete (10–30 s). Switch off vacuum, and ventilate QIAvac 96 manifold.**

   Note: Ensure that ethanol is added to Buffer RPE (see "Important notes before starting", page 35).

9. **Place the RNeasy 96 plate on top of a Square-Well Block. Mark the RNeasy 96 plate for later identification.**

10. **Add another 1 ml of Buffer RPE to each well of the RNeasy 96 plate. Seal the RNeasy 96 plate with AirPore Tape. Load the Square-Well Block and RNeasy 96 plate into the holder, and place the whole assembly in the rotor bucket. Centrifuge at 6000 rpm (~5600 x *g*) for 10 min at room temperature to dry the plate membranes.**

    Centrifugation with sealed plates prevents cross-contamination.

    It is important to dry the RNeasy membrane since residual ethanol may interfere with subsequent reactions. The 10 min spin ensures that residual traces of salt are removed and that no ethanol is carried over during elution.

11. **Remove AirPore Tape. Place RNeasy 96 plate on top of an elution microtube rack containing 1.2 ml elution microtubes.**

12. **To elute the RNA, add 45–70 µl of RNase-free water to each well, and seal the RNeasy 96 plate with a new sheet of AirPore Tape. Incubate for 1 min at room temperature. Then centrifuge at 6000 rpm (~5600 x *g*) for 4 min at room temperature.**

    Note: Make sure to pipet the RNase-free water directly onto the RNeasy membrane. Elution will be incomplete if some of the water sticks to the walls or the O-rings of the RNeasy 96 plate.

13. **Remove AirPore Tape. Repeat the elution step (step 12) once with a second volume of 45–70 µl RNase-free water.**

    Note: Repeating the elution step is required for complete recovery of RNA. The elution volume will be 15 µl less than the volume of RNase-free water added to the membrane, corresponding to the membrane dead volume.

    Use caps provided to seal the microtubes for storage. Store RNA at –20°C or at –70°C.

RNeasy 96 Protocol for RNA Cleanup

III.Using spin technology

1. **Adjust each sample volume to 100 µl with RNase-free water. Add 350 µl Buffer RLT to each sample, and mix by pipetting up and down 3 times with a multichannel pipet.**
2. **Add 250 µl of ethanol (96–100%) to each sample, and mix by pipetting 3 times up and down with a multichannel pipet.**
3. **Place an RNeasy 96 plate on top of a Square-Well Block. Mark the RNeasy 96 plate for later identification.**
4. **Apply the samples from step 2 (700 µl) into the wells of the RNeasy 96 plate.**

   Take care not to wet the rims of the wells to avoid aerosols during centrifugation.
5. **Seal the RNeasy 96 plate with AirPore Tape. Load the Square-Well Block and RNeasy 96 plate into the holder, and place the whole assembly in the rotor bucket. Centrifuge at 6000 rpm (~5600 x g) for 4 min at room temperature.**

   Centrifugation with sealed plates prevents cross-contamination.
6. **DNase digestion (optional)**

   Note: Generally, DNase digestion is not required since the RNeasy 96 silica-membrane technology efficiently removes most of the DNA without DNase treatment. However, further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA. See page 48 for more information and page 18 for details to prepare the DNase I incubation mix.

   A. Remove AirPore Tape. Pipet 80 µl of the DNase I incubation mix (see page 18) directly onto the RNeasy membrane in each well of the RNeasy 96 plate. Seal the plate with a new sheet of AirPore Tape.

      Note: Make sure to pipet the DNase I incubation mix directly onto the RNeasy membrane. DNase digestion will be incomplete if some of the mix sticks to the walls or the O-rings of the RNeasy 96 plate.

   B. Place at room temperature for 15 min. Then proceed with step 7.
7. **Remove AirPore Tape. Add 0.8 ml of Buffer RW1 to each well of the RNeasy 96 plate. Seal the RNeasy 96 plate with a new sheet of AirPore Tape. (If on-membrane DNase digestion was performed in the previous step, wait 5 min before proceeding.) Centrifuge at 6000 rpm (~5600 x g) for 4 min at room temperature.**

   Note: To efficiently remove the DNase following DNase digestion, allow Buffer RW1 to remain on the RNeasy membrane for 5 min before centrifuging the buffer through the membrane.
8. **Place the RNeasy 96 plate on top of a another clean Square-Well Block. Remove AirPore Tape. Add 0.8 ml of Buffer RPE to each well of the RNeasy 96 plate. Seal the RNeasy 96 plate with a new sheet of AirPore Tape. Centrifuge at 6000 rpm (~5600 x g) for 4 min at room temperature.**

   Note: Ensure that ethanol is added to Buffer RPE (see "Important notes before starting", page 35).
9. **Remove AirPore Tape. Add another 0.8 ml of Buffer RPE to each well of the RNeasy 96 plate. Seal the RNeasy 96 plate with a new sheet of AirPore Tape. Centrifuge at 6000 rpm (~5600 x g) for 10 min at room temperature.**

   It is important to dry the RNeasy membrane since residual ethanol may interfere with subsequent reactions. The 10 min-spin ensures that residual traces of salt are removed and that no ethanol is carried over during elution.

10. **Remove AirPore Tape. Place RNeasy 96 plate on top of an elution microtube rack containing 1.2 ml elution microtubes.**

11. **To elute the RNA, add 45–70 µl of RNase-free water to each well, and seal the RNeasy 96 plate with a new sheet of AirPore Tape. Incubate for 1 min at room temperature. Then centrifuge at 6000 rpm (~5600 x *g*) for 4 min at room temperature.**

    Note: Make sure to pipet the RNase-free water directly onto the RNeasy membrane. Elution will be incomplete if some of the water sticks to the walls or the O-rings of the RNeasy 96 plate.

12. **Remove AirPore Tape. Repeat the elution step (step 11) once with a second volume of 45–70 µl RNase-free water.**

    Note: Repeating the elution step is required for complete recovery of RNA. The elution volume will be 15 µl less than the volume of RNase-free water added to the membrane, corresponding to the membrane dead volume.

    Use caps provided to seal the microtubes for storage. Store RNA at –20°C or at –70°C.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or molecular biology applications (see inside front cover for contact information).

	Comments and suggestions
Clogged plate wells	
Too much starting material	Reduce amount of starting material. It is essential to use the correct amount of starting material (see page 12).
Little or no RNA eluted	
a) Too much starting material	Overloading significantly reduces yield. Reduce the amount of starting material (see page 12).
b) Incomplete removal of supernatant	Ensure complete removal of the supernatant after harvesting cells (see protocols).
c) Buffer temperatures too low	With the exception of Buffer RLN (2–8°C), all buffers must be at room temperature throughout the procedure.
Low A_{260}/A_{280} value	Use 10 mM Tris·Cl, not RNase-free water, to dilute the sample before measuring purity (see Appendix B, page 47).
RNA degraded	
a) Inappropriate handling of starting material	Ensure that cells have been properly handled and that the protocol has been performed without interruptions, especially the initial steps involving cell lysis. Cell lysis in the cytoplasmic RNA protocol must be performed on ice whereas cell lysis in the total RNA protocol must be performed at room temperature. See Appendix A (page 45), "Handling and storage of starting material" (page 14), and the "Important notes before starting" for each protocol.
b) RNase contamination	Check for RNase contamination of buffers. Although all buffers have been tested and are guaranteed RNase-free, RNases can be introduced during use. Be certain not to introduce any RNase during the procedure or later handling. See Appendix A (page 45) for general remarks on handling RNA.
DNA contamination in downstream experiments	
a) Optimal procedure not used	The cytoplasmic RNA protocol is recommended for applications where the absence of DNA contamination is critical since the intact nuclei are removed at the start of the procedure. Follow the protocol for isolation of cytoplasmic RNA (see page 10 and cytoplasmic protocol on page 26). Vacuum/spin or spin technology is generally more efficient at removing any DNA contamination. Follow the protocol using vacuum/spin or spin technology (see page 11 and protocols).

	Comments and suggestions
a) No incubation with Buffer RW1 after optional DNase treatment	In subsequent preparations, incubate plate wells for 5 min at room temperature with Buffer RW1 following the DNase-treatment step.
b) No DNase treatment	Follow the optional on-column DNase digest step in the protocol. Alternatively after the RNeasy 96 procedure, DNase digest the eluate containing the RNA. After inactivating DNase by heat treatment, the RNA can be either used directly in the subsequent application without further treatment, or repurified using the RNA cleanup protocol.
c) Elution volume too low	Use elution volumes of 60–70 µl if using vacuum technology or 45–70 µl if using vacuum/spin or spin technology. Repeat the elution step (see protocols).
RNA does not perform well in downstream experiments	
a) Salt carryover during elution	Ensure that Buffer RPE is at room temperature (20 to 30°C). If using vacuum technology, be sure to strike the bottom side of the RNeasy 96 plate repeatedly on a stack of paper towels until no further liquid is released (see protocols). Follow the protocol using vacuum/spin or spin technology (see page 11).
b) Ethanol carryover	During the second Buffer RPE wash, be sure to dry the plate-well membranes by vacuum for 10 min (vacuum technology) or centrifuge the plate at 6000 rpm (~5600 x *g*) for 10 min at room temperature (vacuum/spin or spin technology). If using vacuum technology, be sure to strike the bottom side of the RNeasy 96 plate repeatedly on a stack of paper towels until no further liquid is released (see protocols).
c) Elution volume too low	Use elution volumes of 60–70 µl if using vacuum technology or 45–70 µl if using vacuum/spin or spin technology. Repeat the elution step (see protocols).
d) Vacuum pressure too low	A vacuum source capable of generating a vacuum pressure of –800 to –900 mbar is necessary to achieve efficient RNA binding to the membrane, washing, and elution when using vacuum or vacuum/spin technology (see page 15).

	Comments and suggestions
Low well-to-well reproducibility	
a) Elution volume too low	Use elution volumes of 60–70 µl if using vacuum technology or 45–70 µl if using vacuum/spin or spin technology. Repeat the elution step (see protocols).
b) Vacuum pressure too low	A vacuum source capable of generating a vacuum pressure of –800 to –900 mbar is necessary to achieve efficient RNA binding to the membrane, washing, and elution when using vacuum or vacuum/spin technology (see page 15).

Appendix A: General Remarks on Handling RNA

Handling RNA

Ribonucleases (RNases) are very stable and active enzymes that generally do not require cofactors to function. Since RNases are difficult to inactivate and even minute amounts are sufficient to destroy RNA, do not use any plasticware or glassware without first eliminating possible RNase contamination. Great care should be taken to avoid inadvertently introducing RNases into the RNA sample during or after the isolation procedure. In order to create and maintain an RNase-free environment, the following precautions must be taken during pretreatment and use of disposable and non-disposable vessels and solutions while working with RNA.

General handling

Proper microbiological, aseptic technique should always be used when working with RNA. Hands and dust particles may carry bacteria and molds and are the most common sources of RNase contamination. Always wear latex or vinyl gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin or from dusty laboratory equipment. Change gloves frequently and keep tubes closed whenever possible. Keep isolated RNA on ice when aliquots are pipetted for downstream applications.

Disposable plasticware

The use of sterile, disposable polypropylene tubes is recommended throughout the procedure. These tubes are generally RNase-free and do not require pretreatment to inactivate RNases.

Non-disposable plasticware

Non-disposable plasticware should be treated before use to ensure that it is RNase-free. Plasticware should be thoroughly rinsed with 0.1 M NaOH, 1 mM EDTA followed by RNase-free water (see "Solutions", page 46). Alternatively, chloroform-resistant plasticware can be rinsed with chloroform to inactivate RNases.

Glassware

Glassware should be treated before use to ensure that it is RNase-free. Glassware used for RNA work should be cleaned with a detergent, thoroughly rinsed, and oven baked at 240°C for four or more hours (overnight, if more convenient) before use. Autoclaving alone will not fully inactivate many RNases. Alternatively, glassware can be treated with DEPC* (diethyl pyrocarbonate). Fill glassware with 0.1% DEPC (0.1% in water), allow to stand overnight (12 hours) at 37°C, and then autoclave or heat to 100°C for 15 minutes to eliminate residual DEPC.

Electrophoresis Tanks

Electrophoresis tanks should be cleaned with detergent solution (e.g., 0.5% SDS), thoroughly rinsed with RNase-free water, and then rinsed with ethanol† and allowed to dry.

* *DEPC is a suspected carcinogen and should be handled with great care. Wear gloves and use a fume hood when using this chemical.*

† *Plastics used for some electrophoresis tanks are not resistant to ethanol. Take proper care and check the supplier's instructions.*

Solutions

Solutions (water and other solutions) should be treated with 0.1% DEPC*. DEPC is a strong, but not absolute, inhibitor of RNases. It is commonly used at a concentration of 0.1% to inactivate RNases on glass or plasticware or to create RNase-free solutions and water. DEPC inactivates RNases by covalent modification. Add 0.1 ml DEPC to 100 ml of the solution to be treated and shake vigorously to bring the DEPC into solution. Let the solution incubate for 12 hours at 37°C. Autoclave for 15 minutes to remove any trace of DEPC. DEPC will react with primary amines and cannot be used directly to treat Tris buffers. DEPC is highly unstable in the presence of Tris buffers and decomposes rapidly into ethanol and CO_2. When preparing Tris buffers, treat water with DEPC first, and then dissolve Tris to make the appropriate buffer. Trace amounts of DEPC will modify purine residues in RNA by carboxymethylation. Carboxymethylated RNA is translated with very low efficiency in cell-free systems. However, its ability to form DNA:RNA or RNA:RNA hybrids is not seriously affected unless a large fraction of the purine residues have been modified. Residual DEPC must always be eliminated from solutions or vessels by autoclaving or heating to 100°C for 15 minutes.

Note: RNeasy buffers are guaranteed RNase-free without using DEPC treatment and are therefore free of any DEPC contamination.

** DEPC is a suspected carcinogen and should be handled with great care. Wear gloves and use a fume hood when using this chemical.*

Appendix B: Storage, Quantification, and Determination of Quality of RNA

Storage of RNA

Purified RNA may be stored at –20°C or –70°C in water. Under these conditions, no degradation of RNA is detectable after 1 year.

Quantification of RNA

The concentration of RNA should be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer. To ensure significance, readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 40 µg of RNA per ml (A_{260}=1 ⇒ 40 µg/ml). This relation is valid only for measurements at a neutral pH. Therefore, if it is necessary to dilute the RNA sample, this should be done in a buffer with neutral pH. As discussed below (see "Purity of RNA"), the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity.

When measuring RNA samples, be certain that cuvettes are RNase-free, especially if the RNA is to be recovered after spectrophotometry. This can be accomplished by washing cuvettes with 0.1M NaOH, 1 mM EDTA followed by washing with RNase-free water (see "Solutions", page 46). Use the buffer in which the RNA is diluted to zero the spectrophotometer.

An example of the calculation involved in RNA quantification is shown below:

Volume of RNA sample = 100 µl
Dilution = 20 µl of RNA sample + 180 µl of 10 mM Tris, pH 7.0 (1/10 dilution).
Measure absorbance of diluted sample in a 0.2 ml cuvette (RNase-free).
A_{260} = 0.2

Concentration of RNA sample	= 40 µg/ml x A_{260} x dilution factor = 40 µg/ml x 0.2 x 10 = 80 µg/ml
Total amount	= concentration x volume of sample in ml = 80 µg/ml x 0.1 ml = 8 µg of RNA

Purity of RNA

The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV, such as protein. However, the A_{260}/A_{280} ratio is influenced considerably by pH. Since water is not buffered, the pH and the resulting A_{260}/A_{280} ratio can vary greatly. Lower pH results in a lower A_{260}/A_{280} ratio and reduced sensitivity to protein contamination.* For accurate values, we recommend measuring absorbance in 10 mM Tris·Cl, pH 7.5. Pure RNA has an A_{260}/A_{280} ratio of 1.9–2.1† in 10 mM Tris·Cl, pH 7.5. Always be sure to calibrate the spectrophotometer with the same solution.

For determination of RNA concentration, however, we recommend dilution of the sample in a buffer with neutral pH since the relationship between absorbance and concentration (A_{260} reading of 1 = 40 µg/ml RNA) is based on an extinction coefficient calculated for RNA at neutral pH (see "Quantification of RNA").

* *Wilfinger, W.W., Mackey, M., and Chomczynski, P. (1997) Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. BioTechniques* ***22****, 474.*

† *Values up to 2.3 are routinely obtained for pure RNA (in 10 mM Tris·Cl, pH 7.5) with some spectrophotometers.*

DNA contamination

No currently available purification method can guarantee that RNA is completely free of DNA, even when it is not visible on an agarose gel. To prevent any interference by DNA in RT-PCR applications, such as TaqMan and LightCycler RT-PCR analyses, we recommend working with intron-spanning primers so that genomic DNA will not be amplified. Alternatively, DNA contamination can be detected on agarose gels following RT-PCR by performing control experiments in which no reverse transcriptase is added prior to the PCR step. For sensitive applications, such as differential display, or if it is not practical to use intron-spanning primers in TaqMan RT-PCR analysis, DNase digestion of the purified RNA with RNase-free DNase is recommended.

An optional on-column DNase digest step is included in all protocols. The DNase is efficiently washed away in the subsequent wash steps. Alternatively, after the RNeasy 96 procedure, the eluate containing the RNA can be treated with DNase. The RNA can then be repurified with the RNeasy cleanup protocol, or after heat inactivation of the DNase, the RNA can be used directly in downstream applications.

The cytoplasmic protocol is particularly advantageous in applications where the absence of DNA contamination is critical, since the intact nuclei are removed. Using the cytoplasmic protocol, DNase digestion is generally not required: most of the DNA is removed with the nuclei, and the RNeasy 96 silica-membrane technology efficiently removes nearly all of the remaining small amounts of DNA without DNase treatment. However, further DNA removal may be desirable for certain RNA applications that are sensitive to very small amounts of DNA (e.g., TaqMan RT-PCR analysis with a low-abundant target). Using the cytoplasmic protocol with the optional DNase digestion results in undetectable levels of DNA, even by sensitive TaqMan analysis.

Integrity of RNA

The integrity and size distribution of total RNA purified with RNeasy Kits can be checked by denaturing agarose gel electrophoresis and ethidium bromide staining (see "Appendix E: Protocol for Formaldehyde Agarose Gel Electrophoresis", page 53). The respective ribosomal bands (Table 5) should appear as sharp bands on the stained gel. 28S ribosomal RNA bands should be present at approximately twice the amounts of the 18S RNA band. If the ribosomal bands in a given lane are not sharp, but appear as a smear towards smaller sized RNAs, it is likely that the RNA sample suffered major degradation during preparation.

Table 5. Size of ribosomal RNAs from various sources

Source	rRNA	Size (kb)
Mouse	18S	1.9
	28S	4.7
Human	18S	1.9
	28S	5.0

Appendix C: RNeasy 96 for Real-Time, Quantitative RT-PCR

Overview of real-time, quantitative RT-PCR technology

High-throughput RNA analysis is an increasingly important tool in biomedical research, diagnostics, and drug discovery. Quantitative, real-time, high-throughput RT-PCR is possible with RNA purified with the RNeasy 96 Kit in combination with QuantiTect™ RT-PCR Kits from QIAGEN. The QuantiTect SYBR® Green RT-PCR Kit is designed for RNA quantification on any real-time thermal cycler using the fluorescent dye SYBR Green I. The QuantiTect Probe RT-PCR Kit provides minimal optimization on any real-time thermal cycler using sequence-specific probes. This includes the use of dual-labeled (TaqMan) probes, FRET probes (including LightCycler hybridization probes), and Molecular Beacons.

This appendix describes some of these technologies and provides guidelines for setting up standard curves for real-time quantitative RT-PCR analyses. For more information, see the *QuantiTect Probe RT-PCR Handbook* and the *QuantiTect SYBR Green RT-PCR Handbook*.

Dual-labeled probes

Dual-labeled probes, including TaqMan probes, are sequence-specific oligonucleotides with a fluorophore and a quencher dye attached (Figure 3). The fluorophore is at the 5' end of the probe, and the quencher dye is usually located at the 3' end or internally. During the extension phase of PCR, the probe is cleaved by the 5' → 3' exonuclease activity of *Taq* DNA polymerase or HotStarTaq DNA Polymerase, separating the fluorophore and the quencher dyes. This results in detectable fluorescence that is directly proportional to the amount of accumulated target DNA. QIAGEN Operon offers a large number of dual-labeled probes with different fluorescent reporters and quenchers (see **www.operon.com**).

Dual-Labeled Probe Principle

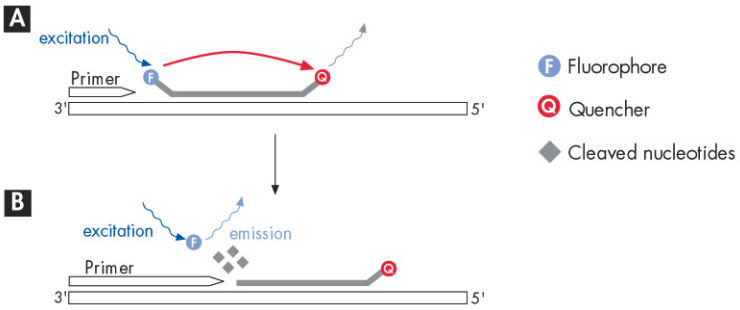

Figure 3. *Schematic diagram of the principle of dual-labeled probes in quantitative, real-time RT-PCR. A Both the dual-labeled probe and the PCR primers anneal to the target sequence during the PCR annealing step. The proximity of the fluorescent reporter with the quencher prevents the reporter from fluorescing. B During the PCR extension step, Taq DNA polymerase or HotStarTaq DNA Polymerase extend the primer. When the enzyme reaches the dual-labeled probe, its 5'→3' exonuclease activity cleaves the fluorescent reporter from the probe. The fluorescent signal from the free reporter is measured.*

FRET probes

PCR with fluorescent resonance energy transfer (FRET) probes, such as LightCycler hybridization probes, uses 2 labeled oligonucleotide probes that bind to the PCR product in a head-to-tail fashion (Figure 4). When the 2 oligonucleotides bind, their fluorophores come into close proximity, allowing energy transfer from a donor to an acceptor fluorophore. This causes fluorescence that is proportional to the amount of product. Unlike dual-labeled probes, FRET probes are not cleaved during the reaction; they can bind to target again in the next PCR cycle. QIAGEN Operon offers a large number of FRET probes

with different fluorescent reporters and quenchers and is a licensed supplier of LC-Red 640 for FRET probes (see **www.operon.com**).

FRET Probe Principle

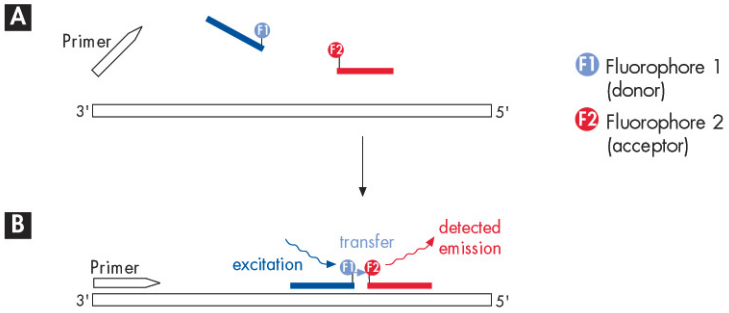

Figure 4. *Schematic diagram of the principle of FRET probes in quantitative, real-time RT-PCR.* ***A*** *When not bound to their target sequence, no fluorescent signal from the acceptor flurophore is detected.* ***B*** *During the PCR annealing step, both FRET probes hybridize to the target sequence. This brings the donor and acceptor fluorophore into close proximity, allowing energy transfer between the fluorophores and resulting in a fluorescent signal from the acceptor fluorophore that is detected. The amount of signal is proportional to the amount of target sequence, and is measured in real time to allow quantification of the amount of target sequence.*

Molecular Beacons

Molecular Beacons are dual-labeled with a 3' quencher and a 5' fluorophore. The probes are designed so that the ends are complementary. When the probe is in solution, the ends bind together and form a stem–loop structure with the fluorophore and the quencher near each other (Figure 5). This effectively quenches the fluorescent signal. When the probe finds its target and binds to it, the stem opens and the fluorophore and the quencher separate. This generates a fluorescent signal proportional to the amount of PCR product. QIAGEN Operon is a licensed supplier of Molecular Beacons, with a large number of different fluorescent reporters and quenchers (see **www.operon.com**).

Molecular Beacon Principle

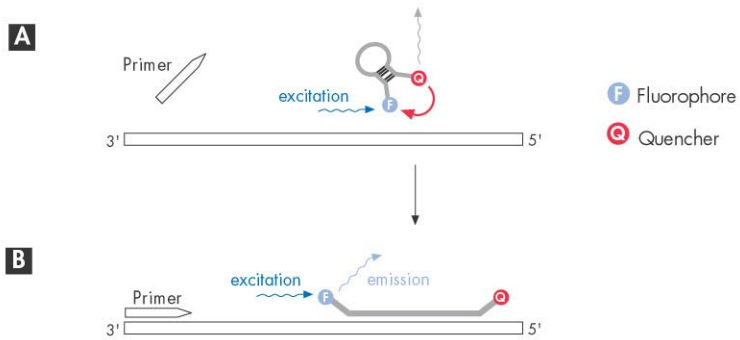

Figure 5. *Schematic diagram of the principle of Molecular Beacons in quantitative, real-time RT-PCR.* ***A*** *When not bound to its target sequence, the Molecular Beacon forms a hairpin structure. The proximity of the fluorescent reporter with the quencher prevents the reporter from fluorescing.* ***B*** *During the PCR annealing step, the Molecular Beacon probe hybridizes to its target sequence. This separates the fluorescent dye and reporter, resulting in a fluorescent signal. The amount of signal is proportional to the amount of target sequence, and is measured in real time to allow quantification of the amount of target sequence.*

Quantification on real-time thermal cyclers

Quantification is based on the threshold cycle, where the amplification plot crosses a defined fluorescence threshold. Comparison of the threshold cycles provides a highly sensitive measure of relative template concentration in different samples. Figure 6 shows an example of real-time analysis using dual-labeled probes in TaqMan analysis. Monitoring during the early cycles, when PCR fidelity is at its highest, provides precise data for accurate quantification.

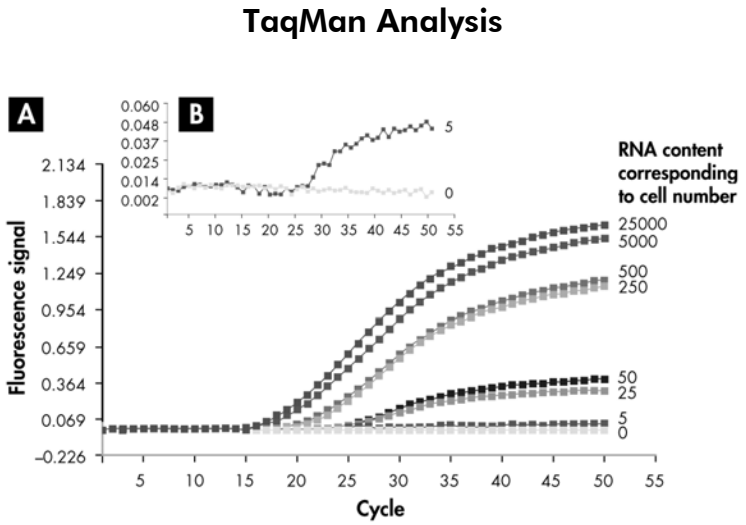

Figure 6. *TaqMan quantitative RT-PCR analysis of cytoplasmic RNA isolated from HeLa cells using the RNeasy 96 Kit.*
A *Real-time RT-PCR analysis of β-actin mRNA was performed with cytoplasmic RNA isolated from 100 to 5 x 10^5 HeLa cells, using 1/20 of each eluate (corresponding to RNA from 5 to 25,000 cells) in a single-tube protocol. The number of PCR cycles needed to detect the amplicon (threshold cycle) is a highly sensitive measure of relative template concentration.*
B *Detail of (A). Signals were analyzed with cytoplasmic RNA corresponding to 5 cells.*

For transcription analysis and quantification, quantitative RT-PCR assays require the highest-quality RNA. TaqMan technology was used in the development and evaluation of the RNeasy 96 Kit, and RNA purified with the RNeasy 96 Kit continues to be thoroughly tested by TaqMan and other real-time analyses. The RNeasy 96 Kit is the only high-throughput total cellular RNA purification system providing RNA that meets stringent TaqMan standards.

Guidelines for quantitative RT-PCR analysis

Quantitative, real-time RT-PCR analysis can be carried out in a two-tube or one-tube format. In two-tube RT-PCR analysis, the reverse-transcription reaction and PCR quantification are performed sequentially in two separate reactions. This can be carried out using Omniscript RT for reverse transcription, followed by PCR using the QuantiTect PCR Kits. For one-tube RT-PCR analysis, using the QuantiTect RT-PCR Kits, both reactions are performed in the same tube on a real-time thermal cycler. Generally, one-tube systems are more commonly used. Some guidelines for setting up quantitative, real-time RT-PCR analysis and determining the linear range of the system are given below.

1. Isolate RNA from cells following one of the RNeasy 96 Protocols for Isolation of Total RNA or Cytoplasmic RNA. For best results, we recommend using either vacuum/spin or spin technology. For the elution steps, elute twice with 45–60 µl RNase-free water. The final volume eluted should be approximately 75–105 µl.
2. For quantitative results, the amount of input RNA must be within the linear response range of the real-time assay, which may vary with the primers used and the transcripts assayed. In order to determine the optimal linear range of input RNA for a specific system, run a series of trial assays with 1, 2, 4, 6, 8, and 10 µl of an RNeasy 96 eluate in a 25 µl reaction volume.* For statistical significance, we recommend assaying each volume in triplicate and repeating the (triplicated) series of assays at least once.

* *The free sample volume in a 25 µl one-tube, real-time RT-PCR analysis is typically 9–10 µl. For a 50 µl assay, with approximately twice the free sample volume, we recommend using 1, 3, 6, 9, 12, 15, 18, and 20 µl.*

3. Plot the resulting threshold cycle against the logarithm of the eluate volume. Figure 7 shows an example of such an experiment, with a linear response over the entire range. Note that, for some systems, the linear response will not cover the full range. Volumes outside the linear range will not yield quantitative results.

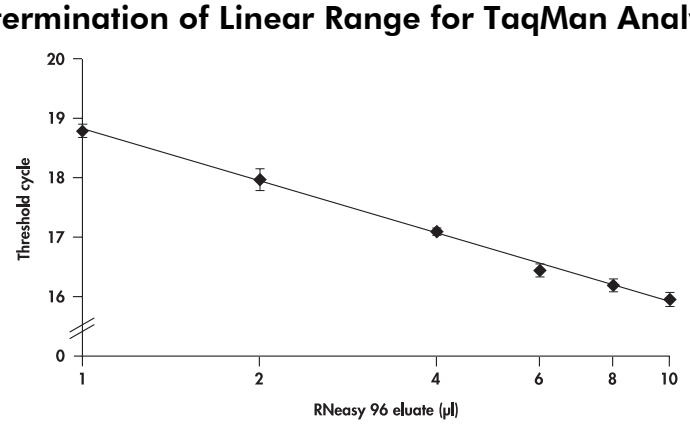

Figure 7. *Linearity of RNeasy 96 RNA isolation for one-tube β-actin RT-PCR analysis using dual-labeled (TaqMan) probes. Total RNA was isolated from 5 x 10^4 HeLa cells with the RNeasy 96 Kit using vacuum/spin technology. RNA was eluted in 2 x 60 µl RNase-free water. RT-PCR TaqMan analysis of β-actin mRNA was performed in triplicate using 1, 2, 4, 6, 8, and 10 µl of the RNeasy 96 eluate in a 25 µl reaction volume, and the entire triplicated series was repeated three times. The mean of the threshold cycle for each volume is presented here, plotted against the logarithm of the volume. Error bars represent the σ_{n-1} standard deviation. The linear response range covers all volumes from 1 to 10 µl.*

Appendix D: Guidelines for RT-PCR

To perform PCR using RNA as a starting template, the RNA must first be reverse transcribed into cDNA in a reverse transcription (RT) reaction. RT and PCR can either be carried out sequentially in the same tube (one-step RT-PCR), or an aliquot of the finished RT reaction can be used in a separate PCR (two-step RT-PCR).

Since the same primers are used in both the RT and the PCR reactions, one-step RT-PCR requires gene-specific primers. For this application, QIAGEN offers the QIAGEN OneStep RT-PCR Kit. Two-step RT-PCR is generally carried out using oligo-dT primers in the RT step and gene-specific primers in the PCR step. General guidelines for two-step RT-PCR are presented in Table 6 below.

Table 6. General guidelines for performing two-step RT-PCR

Reverse transcription:	QIAGEN offers Omniscript™ and Sensiscript™ RT Kits for reverse transcription. Omniscript RT is specially designed for all reverse transcription with any amount of RNA from 50 ng to 2 µg per reaction. Sensiscript RT is optimized for use with very small amounts of RNA (1 pg – 50 ng). Follow the detailed protocol in the accompanying handbook, or, when using an enzyme from another supplier, follow the supplier's instructions. The following guidelines may be helpful. • Mix the following reagents in a microcentrifuge tube: 2.0 µl 10x Buffer RT 2.0 µl dNTP Mix (5 mM each dNTP) 2.0 µl oligo-dT primer (10 µM) 1.0 µl RNase inhibitor (10 units/µl) 1.0 µl Omniscript or Sensiscript RT* template RNA (up to 2 µg with Omniscript RT or up to 50 ng with Sensiscript RT) Add RNase-free water to a final volume of 20 µl. • Incubate at 37°C for 60 min.*
PCR:	• Add an aliquot of the finished reverse-transcription reaction to the PCR mix. (No more than 1/5 of the final PCR volume should derive from the finished reverse-transcription reaction.) • Carry out PCR with *Taq* DNA polymerase as recommended by the supplier. (We have consistently obtained excellent results using *Taq* DNA Polymerase or HotStarTaq™ DNA Polymerase from QIAGEN.)

** For other enzymes, refer to supplier's instructions.*

Appendix E: Protocol for Formaldehyde Agarose Gel Electrophoresis

The following protocol for formaldehyde agarose (FA) gel electrophoresis is routinely used at QIAGEN and gives enhanced sensitivity for gel and subsequent analysis (e.g., northern blotting). A key feature is the concentrated RNA loading buffer that allows a larger volume of RNA sample to be loaded onto the gel than conventional protocols (e.g., Sambrook et al., eds. (1989) *Molecular cloning — a laboratory manual*, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

FA gel preparation

To prepare FA gel (1.2% agarose) of size 10 x 14 x 0.7 cm, mix:

1.2 g agarose

10 ml 10x FA gel buffer (see composition below)

add RNase-free water to 100 ml

If smaller or larger gels are needed, adjust the quantities of components proportionately.

Heat the mixture to melt agarose. Cool to 65°C in a water bath. Add 1.8 ml of 37% (12.3 M) formaldehyde* and 1 µl of a 10 mg/ml ethidium bromide* stock solution. Mix thoroughly and pour onto gel support. Prior to running the gel, equilibrate in 1x FA gel running buffer (see composition below) for at least 30 min.

RNA sample preparation for FA gel electrophoresis

Add 1 volume of 5x loading buffer (see composition below) to 4 volumes of RNA sample (for example 10 µl of loading buffer and 40 µl of RNA) and mix.

Incubate for 3–5 min at 65°C, chill on ice, and load onto the equilibrated FA gel.

Gel running conditions

Run gel at 5–7 V/cm in 1x FA gel running buffer.

Composition of FA gel buffers

10x FA gel buffer

200 mM 3-[N-morpholino]propanesulfonic acid (MOPS) (free acid)

50 mM sodium acetate

10 mM EDTA

pH to 7.0 with NaOH

1x FA gel running buffer

100 ml 10x FA gel buffer

20 ml 37% (12.3 M) formaldehyde*

880 ml RNase-free water

** Toxic and/or mutagenic. Take appropriate safety measures.*

5x RNA loading buffer

16 µl saturated aqueous bromophenol blue solution*

80 µl 500 mM EDTA, pH 8.0

720 µl 37% (12.3 M) formaldehyde†

2 ml 100% glycerol

3.084 ml formamide

4 ml 10 x FA gel buffer

RNase-free water to 10 ml

Stability: approximately 3 months at 4°C

** To make a saturated solution, add solid bromophenol blue to distilled water. Mix and continue to add more bromophenol blue until no more will dissolve. Centrifuge to pellet the undissolved powder, and carefully pipet the saturated supernatant.*

† Toxic and/or mutagenic. Take appropriate safety measures.

Appendix F: Equipment and Reagent Suppliers*

DNase I, RNase-free, can be purchased from:

- QIAGEN (as part of the RNase-Free DNase Set)
- Worthington Biochemical Corp.
- Amersham Biosciences
- Roche Molecular Biochemicals (formerly Boehringer Mannheim Corp.)
- Stratagene

RNase inhibitors can be purchased from:

- Promega
- Applied Biosystems
- Pharmacia
- Stratagene

Matrix Impact or Multi-8 Electrapette can be purchased from:

- Matrix Technologies Corporation (**www. matrixtechcorp.com**)

Vacuum pumps (18 liter/min) can be purchased from:

- In the USA: KNF Neuberger Inc., 2 Black Forest Road, Edgebrook Park, Trenton, NJ 08691-9428 Telephone: 1-609-890-8889
- In Germany: KNF Neuberger GmbH, Alter Weg 3, D-79112 Freiburg Telephone: (49) 7664-5909-0

** This is not a complete list of suppliers and does not include many important vendors of biological supplies.*

Ordering Information

Product	Contents	Cat. No.
RNeasy 96 Kit		
RNeasy 96 Kit* (4)	For 4 x 96 total RNA preps: 4 RNeasy 96 Plates, Elution Microtubes (1.2 ml), Caps, RNase-free Reagents and Buffers	74181
RNeasy 96 Kit* (12)	For 12 x 96 total RNA preps: 12 RNeasy 96 Plates, Elution Microtubes (1.2 ml), Caps, RNase-free Reagents and Buffers	74182
Accessories		
QIAvac 96	Vacuum manifold for processing QIAGEN 96-well plates: includes QIAvac 96 Top Plate, Base, Waste Tray, Plate Holder	19504
Vacuum Regulator	For use with QIAvac manifolds	19530
Elution Microtube Adapter	Adapter for using Elution Microtube Racks on the QIAvac 96 and the BioRobot vacuum manifold	Inquire
Centrifuge 4-15C†	Universal laboratory centrifuge with brushless motor	Inquire
Centrifuge 4K15C†	Universal refrigerated laboratory centrifuge with brushless motor	Inquire
Plate Rotor 2 x 96‡	Rotor for 2 QIAGEN 96 plates, for use with QIAGEN Centrifuges	81031
Buffer RLT	220 ml RNeasy Lysis Buffer for 6 RNeasy 96 plates	79216
Square-Well Blocks (24)	96-well blocks with 2.2 ml wells, 24 blocks per case	19573
AirPore Tape Sheets (50)	Microporous tape sheets for covering 96-well blocks: 50 sheets per pack	19571
Tape Pads (5)	Adhesive tape sheets for sealing multiwell plates and blocks: 25 sheets per pad, 5 pads per pack	19570
Elution Microtubes (racked)	Nonsterile polypropylene tubes (1.2 ml), 960 in racks of 96	Inquire
Elution Microtubes (loose)	Nonsterile polypropylene tubes (1.2 ml), 960 in strips of 8, loose in bag	Inquire
Caps for Elution Microtubes (120 x 8)	Nonsterile polypropylene caps for elution microtubes (1.2 ml), 960 in strips of 8	Inquire

* *Requires use of either QIAvac 96 or the QIAGEN 96-Well-Plate Centrifugation System.*

† *Centrifuges 4-15C and 4K15C are not available in all countries. Specific formats are available in Japan. Please inquire.*

‡ *The Plate Rotor 2 x 96 is available exclusively from QIAGEN and its distributors. Under the current liability and warranty conditions, the rotor may only be used in Centrifuges 4-15C and 4K15C from QIAGEN, and freely programmable models of centrifuges 4-15, 4K15, 6-10, 6K10, 6-15, and 6K15 from Sigma Laborzentrifugen GmbH.*

Product	Contents	Cat. No.
Related products		
RNeasy 96 BioRobot® Kits — for automated, high-throughput RNA isolation from cells		
RNeasy 96 BioRobot 9604 Kit (12)	For 12 x 96 total and cytoplasmic RNA preps on the BioRobot 9604 configuration C: 12 RNeasy 96 Plates, Elution Microtubes (1.2 ml), Caps, Square-Well Blocks, RNase-free Reagents and Buffers	967142
RNeasy 96 BioRobot 8000 Kit (12)	For 12 x 96 total and cytoplasmic RNA preps on the BioRobot 8000: 12 RNeasy 96 Plates, Elution Microtubes (1.2 ml), Caps, Square-Well Blocks, RNase-free Reagents and Buffers	Inquire
QIAamp 96 Virus BioRobot Kit — for automated high-throughput purification of viral RNA and DNA from cell-free body fluids		
QIAamp 96 Virus BioRobot Kit (12)	For 12 x 96 nucleic acid preps: 12 QIAamp 96 Plates, RNase-free Buffers, QIAGEN Protease, AirPore Tape Sheets, Tape Pad, S-Blocks, Racks with Collection Microtubes (1.2 ml), Carrier RNA, Caps	965642
BioRobot 9604* — for automated nucleic acid purification in molecular diagnostics and research		
BioRobot 9604	System includes: robotic workstation with 4 dilutor drives; microprocessor-controlled vacuum pump; vacuum manifold; High-Speed Pipetting System; Tip-Change System; QIAsoft™ 3.0 Operating System, Basic Edition; computer, cables; installation and training; 1-year warranty on parts and labor	900300
BioRobot 8000* — for high-throughput, walk-away nucleic acid purification		
BioRobot 8000	System includes: robotic workstation comprised of 8 dilutor units and selected system components; variable spacing system; QIAsoft 4.1 Operating System; computer; installation and training; 1 year warranty on parts and labor	900500
DNeasy® 96 Tissue Kit† — for high-throughput DNA isolation from animal tissues and cells		
DNeasy 96 Tissue Kit (4)	For 4 x 96 DNA minipreps: 4 DNeasy 96 Plates, Proteinase K, Buffers, Square-Well Blocks, AirPore Tape Sheets, Collection Microtubes (1.2 ml), Caps, 96-well Plate Registers	69581
QIAamp 96 DNA Blood Kit† — for high-throughput DNA isolation from blood and body fluids		
QIAamp 96 DNA Blood Kit (4)	For 4 x 96 DNA preps: 4 QIAamp 96 Plates, QIAGEN Protease, Reagents, Buffers, Lysis Blocks, Tape Pads, Collection Vessels	51161

** QIAGEN Robotic Systems are not available in all countries; please inquire.*

† Larger kit sizes available; please inquire. Requires use of the QIAGEN 96-Well-Plate Centrifugation System.

Product	Contents	Cat. No.
RNeasy Kits — for total RNA isolation from animal cells or tissues, yeast, or bacteria		
RNeasy Mini Kit (50)*	50 RNeasy Mini Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	74104
RNeasy Midi Kit (10)*	10 RNeasy Midi Spin Columns, Collection Tubes (15 ml), RNase-free Reagents and Buffers	75142
RNeasy Maxi Kit (12)	12 RNeasy Maxi Spin Columns, Collection Tubes (50 ml), RNase-free Reagents and Buffers	75162
RNeasy Plant Kit — for total RNA isolation from plants and fungi		
RNeasy Plant Mini Kit (20)*	20 RNeasy Mini Spin Columns, 20 QIAshredder™ Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	74903
QIAamp® RNA Blood Mini Kit — for total cellular RNA isolation from whole human blood		
QIAamp RNA Blood Mini Kit (50)*	50 QIAamp Mini Spin Columns, 50 QIAshredder Spin Columns, Collection Tubes (1.5 ml and 2 ml), RNase-free Reagents and Buffers	52304
RNase-Free DNase Set — for DNase digestion during RNA purification		
RNase-Free DNase Set (50)	1500 units RNase-free DNase I, RNase-free Buffer, and RNase-free water for 50 RNA minipreps	79254
Omniscript RT Kit — for reverse transcription using ≥50 ng RNA		
Omniscript RT Kit (10)*	For 10 reverse-transcription reactions: 40 units Omniscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix,† RNase-free water	205110
Sensiscript RT Kit — for reverse transcription using <50 ng RNA		
Sensiscript RT Kit (50)*	For 50 reverse-transcription reactions: Sensiscript Reverse Transcriptase, 10x Buffer RT, dNTP Mix,† RNase-free water	205211
QIAGEN OneStep RT-PCR Kit — for easy and sensitive one-step RT-PCR		
QIAGEN OneStep RT-PCR Kit (25)*	For 25 reactions: QIAGEN OneStep RT-PCR Enzyme Mix, 5x QIAGEN OneStep RT-PCR Buffer,‡ dNTP Mix,§ 5x Q-Solution, RNase-free water	210210
***Taq* DNA Polymerase — for standard and specialized PCR applications**		
Taq DNA Polymerase (250 U)	250 units *Taq* DNA Polymerase, 10x PCR Buffer,¶ 5x Q-Solution, 25 mM $MgCl_2$	201203
HotStarTaq DNA Polymerase — for highly specific hot-start PCR		
HotStarTaq DNA Polymerase (250 U)	250 units HotStarTaq DNA Polymerase, 10x PCR Buffer,¶ 5x Q-Solution, 25 mM $MgCl_2$	203203

* *Larger kit sizes available; please inquire.*

† *Contains 5 mM of each dNTP*

‡ *Contains 12.5 mM $MgCl_2$*

§ *Contains 10 mM of each dNTP*

¶ *Contains 15 mM $MgCl_2$*

Product	Contents	Cat. No.
QuantiTect SYBR Green PCR and RT-PCR Kits — for quantitative, real-time PCR and RT-PCR using SYBR Green		
QuantiTect SYBR Green PCR Kit (200)	For 200 x 50 µl reactions: 3 x 1.7 ml QuantiTect SYBR Green PCR Master Mix;* 2 x 2.0 ml RNase-free water	204143
QuantiTect SYBR Green RT-PCR Kit (200)	For 200 x 50 µl reactions: 3 x 1.7 ml QuantiTect SYBR Green RT-PCR Master Mix;* 1 x 100 µl QuantiTect RT Mix; 2 x 2.0 ml RNase-free water	204243
QuantiTect Probe PCR and RT-PCR Kits — for quantitative, real-time PCR and RT-PCR using sequence-specific probes		
Quanti Tect Probe PCR Kit (200)	For 200 x 50 µl reactions: 3 x 1.7 ml QuantiTect Probe PCR Master Mix;† 2 x 2.0 ml RNase-free water	204343
QuantiTect Probe RT-PCR Kit (200)	For 200 x 50 µl reactions: 3 x 1.7 ml QuantiTect Probe RT-PCR Master Mix;† 1 x 100 µl QuantiTect RT Mix; 2 x 2.0 ml RNase-free water	204443
QIAGEN Operon Oligonucleotide Synthesis Service — high-quality oligos, modified oligos, and longmers		
Oligonucleotide Synthesis Service	Custom-made oligonucleotides and a wide range of modified oligos, including Molecular Beacons, dual-labeled probes, FRET probes, and many more	Inquire

* *Contains 5 mM $MgCl_2$*

† *Contains 8 mM $MgCl_2$*

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